Chapter 5

In vitro Tissue Culture, a Tool for the Study and Breeding of Plants Subjected to Abiotic Stress Conditions

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Additional information is available at the end of the chapter

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1. Introduction

Abiotic stress factors are the main limitation to plant growth and yield in agriculture. Among them, drought stress caused by water deficit, is probably the most impacting adverse condition and the most widely encountered by plants, not only in crop fields but also in wild environments. According to published statistics, the percentage of drought-affected land area in the world in 2000 was double that of 1970 [1].

Another major environmental factor that limits crop productivity, mainly in arid and semi-arid regions is high salinity. Approximately 19.5% of the irrigated soils in the world have elevated concentrations of salts either in the soil or in the irrigation water [2], damaging both the economy and the environment [3, 4]. The deleterious effects of salinity on plant growth are associated with low osmotic potential of soil solution (water stress), nutritional imbalance, specific ion effect (salt stress), or a combination of these factors [5].

Abiotic stress leads to a series of morphological, physiological, biochemical, and molecular changes that adversely affect plant growth and productivity [6]. Drought, salinity, extreme temperatures, and oxidative stress are often interconnected, and may induce similar cellular damage (for more details see [7]).

During the course of its evolution, plants have developed mechanisms to cope with and adapt to different types of abiotic and biotic stress. Plants face adverse environmental conditions by regulating specific sets of genes in response to stress signals, which vary depending on factors such as the severity of stress conditions, other environmental factors, and the plant species [8].

The sensing of these stresses induces signaling events that activate ion channels, kinase cascades, production of reactive oxygen species, and accumulation of hormones [9].

These signals ultimately induce expression of specific genes that lead to the assembly of the overall defense reaction. In contrast to plant resistance to biotic stresses, which is mostly dependent on monogenic traits, the genetically complex responses to abiotic stresses are multigenic, and thus more difficult to control and engineer [10].

The conventional breeding programs are being used to integrate genes of interest from inter crossing genera and species into the crops to induce stress tolerance. However, in many cases, these conventional breeding methods have failed to provide desirable results [11].

In recent decades, the use of techniques based on *in vitro* plant tissue culture, has made possible the development of biotechnological tools for addressing the critical problems of crop improvement for sustainable agriculture. Among the available biotechnological tools for crop breeding, genetic engineering based on introgression of genes that are known to be involved in plant stress response and *in vitro* selection through the application of selective pressure in culture conditions, for developing stress tolerant plants, have proved to be the most effective approaches [12].

On the other hand, it is often difficult to analyze the response of plants to different abiotic stresses in the field or in greenhouse conditions, due to complex and variable nature of these stresses. *In vitro* tissue culture-based tools have also allowed a deeper understanding of the physiology and biochemistry in plants cultured under adverse environmental conditions [13].

In this work, the progress made towards the development of abiotic stress-tolerant plants through tissue culture-based approaches is described. The achievements in the better understanding of physiological and biochemical changes in plants under *in vitro* stress conditions are also reviewed.

2. Somaclonal variation

Somaclonal variation is defined as the genetic and phenotypic variation among clonally propagated plants of a single donor clone. It is well known that genetic variations occur in undifferentiated cells, isolated protoplasts, calli, tissues and morphological traits of regenerated plants. The cause of variation is mostly attributed to changes in the chromosome number and structure. Generally, the term somaclonal variation is used for genetic variability present among all kinds of cells/plants obtained from cells cultured *in vitro* [14].

Plants regenerated from tissue and cell cultures show heritable variation for both qualitative and quantitative traits. Somaclonal variation caused by the process of tissue culture is also called tissue culture-induced variation to more specifically define the inducing environment [15]. The occurrence of uncontrolled and spontaneous variation during the culture process is an unexpected and mostly undesired phenomenon when plants are micropropagated at the commercial scale [16]. However, apart from these negative effects, its usefulness in crop breeding through creation of novel variants has been extensively reported [17]. Induced somaclonal variation can be used for genetic manipulation of crops with polygenic traits

[18]. The new varieties derived from *in vitro* tissue culture could exhibit disease resistance and improvement in quality as well as better yield [19].

Somaclonal variants can be detected using various techniques which are broadly categorized as morphological, physiological/biochemical and molecular detection techniques. There are two main approaches for the isolation of somaclonal variants: screening and cell selection.

Screening involves the observation of a large number of cells or regenerated plants for the detection of variant individuals. Mutants for several traits can be far more easily isolated from cell cultures than from whole plant populations. This is because a large number of cells can be easily and effectively screened for mutant traits. Screening of as many plants would be very difficult, ordinarily impossible [17]. Mutants can be effectively selected for disease resistance, improvement of nutritional quality, adaptation to stress conditions, e.g., saline, soils, low temperature, toxic metals, resistance to herbicides and to increase the biosynthesis of plant products used for medicinal or industrial purposes. Screening has been profitably and widely employed for the isolation of cell clones that produce higher quantities of certain biochemicals [20].

In the cell selection approach, a suitable pressure is applied to permit the preferential survival/growth of variant cells. Selection strategies have been successfully developed for the recovery of genotypes resistant to various toxins, herbicides, high salt concentration etc. [21]. When the selection pressure allows only the mutant cells to survive or divide, it is called positive selection. On the other hand, in the case of negative selection, the wild type cells divide normally and therefore are killed by a counter selection agent, e.g., 5-Bromodeoxyuridine, or arsenate. The mutant cells are unable to divide as a result of which they escape the counter selection agent. These cells are subsequently rescued by removal of the counter selection agent [11].

3. *In vitro* selection of plants tolerant to abiotic stress

Many studies have reported that the *in vitro* culture alone or combined with mutagenesis, induced with physicochemical or biological agents, can be exploited to increase genetic variability and mutants, as a potential source of new commercial cultivars [22]. *In vitro* culture environments can be mutagenic and plants regenerated from organ cultures, calli, protoplasts and via somatic embryogenesis sometimes exhibit phenotypic and/or genotypic variations [22].

It is important to point that tissue culture increases the efficiency of mutagenic treatments and allows handling of large populations and rapid cloning of selected variants [17]. The similarities of the effects induced by the stress in the plant cultured *in vitro* and *in vivo* conditions suggest that the *in vitro* system can be used as an alternative to field evaluations for studying the general effect of water-stress on plant growth and development.

The most widely used method for the selection of genotypes tolerant to abiotic stress is the *in vitro* selection pressure technique. This is based on the *in vitro* culture of plant cells,

tissues or organs on a medium supplemented with selective agents, allowing selecting and regenerating plants with desirable characteristics. In table 1 a list of species in which this technique has been successfully applied to obtain genotypes with increased resistance to different abiotic stresses is shown.

Plant species	stress	References
Chrysanthemum morifolium (chrysanthemum)	salt	[66]
Brassica napus (rapeseed)	salt	[67]
Citrus aurantium (sour orange)	salt	[68]
Lycopersicon esculentum (tomato)	salt	[69]
Dendrocalamus strictus (bamboo)	salt	[11]
Ipomoea batatas (sweet potato)	salt	[70]
Saccharum sp. (sugarcane)	salt	[71]
Solanum tuberosum (potato)	salt	[32], [72]
Triticum aestivum (wheat)	salt	[21], [73]
Arachis hypogaea (groundnut)	drought	[74]
Brassica juncea (indian mustard)	drought	[75]
Prunus avium (colt cherry)	drought	[72]
Saccharum sp. (sugarcane)	drought	[76]
Oryza sativa (rice)	drought/chilling/Al	[77]
Triticum aestivum (wheat)	drought/frost	[73]
Glycine max (soybean)	Al	[78]
Setaria italica (millet)	Zn	[79]

Table 1. *In vitro* selection for increased resistance to abiotic stresses.

The most important successes on this respect are described below:

3.1. *In vitro* selection of salt-tolerant plants

The problem of soil salinity has been aggravated during the last decades as a consequence of some agricultural practices such as irrigation and poor drainage systems. As described in the introduction, it has been estimated that around 20 % of the irrigated land in the world is affected by salinity, and it is expected that the increase of salinization in agricultural fields will reduce the land available for cultivation by 30% in the next 25 years and up to 50% by the year 2050 [23].

The *in vitro* selection pressure technique has been effectively utilized to induce tolerance to salt stress in plants through the use of salts as a selective agent, allowing the preferential survival and growth of desired genotypes. This approach has being done using a number of plant materials (callus, suspension cultures, somatic embryos, shoot cultures, etc.) which has been screened for variation in their ability to tolerate relatively high levels of salt in the culture media. In most of the studies, the salt used has been NaCl [24].

Several researchers have compared the response of other Cl^- and SO_4^{2-} salts including KCl, Na_2SO_4, and $MgSO_4$ during *in vitro* screening. This use of multiple salts as a selection pressure parallels the salinity under field conditions and may be a better choice [11].

3.2. *In vitro* selection of drought-tolerant plants

Drought is a major abiotic stress which causes important agricultural losses, mainly in arid and semiarid areas. Drought stress causes moisture depletion in soil and water deficit with a decrease of water potential in plant tissues. *In vitro* culture has been used to obtain drought-tolerant plants assuming that there is a correlation between cellular and *in vivo* plant responses [25]. During the last years, *in vitro* selection for cells exhibiting increased tolerance to water or drought stress has been reported (Table 1).

Polyethylene glycol (PEG), sucrose, mannitol or sorbitol have been used by several workers as osmotic stress agents for *in vitro* selection [25; 26] However, PEG has been the most extensively used to stimulate water stress in plants. This compound of high molecular weight is a non-penetrating inert osmoticum that reduces water potential of nutrient solutions without being taken up by the plant or being phytotoxic [26]. Because PEG does not enter the apoplast, water is withdrawn not only from the cell but also from the cell wall. Therefore, PEG solutions mimic dry soil more closely than solutions of compounds with low molecular weights, which infiltrate the cell wall with solute [27].

Besides salt and drought, a few reports are also available for the development of plants tolerant to other abiotic stress (metal, chilling, UV and frost) through *in vitro* selection (Reviewed in [11]).

4. Characterization of salt- or drought-tolerant plants during *in vitro* selection

A second step in the process of obtaining genotypes more tolerant to a particular stress condition is the characterization of the regenerants that survive to the imposed pressure selection under *in vitro* conditions. Salinity and drought affect many physiological processes such as reductions of cell growth, leaf area, biomass and yield. The activation of the plant antioxidant defense system has been positively associated with salt and drought tolerance [11], and the same pattern has been confirmed on *in vitro* cultures [28]. Therefore, by measuring antioxidant activities *in vitro*, a rapid preliminary selection of tolerant genotypes could be performed. In fact, different authors have determined the main antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR) [29].

Lipids play an important role as the structural constituent of most of the cellular membranes [30]. Moreover, often there is no need of intact plants to perform the initial selection, as in the case of callus culture that can be used as a plant material for the selection of tolerant genotypes. As an example it is well known that free radical-induced peroxidation of lipid membrane is a sign of stress-induced damage at cellular level. Therefore, the level of

malonyldialdehyde, produced during peroxidation of membrane lipids, is often used as an indicator of oxidative damage [31]. It has been reported that selected callus lines of *Solanum tuberosum* subjected to NaCl showed an increase in lipid peroxidation in comparison with salt tolerant lines [32].

For overcoming salt or drought stress, plants have developed protective mechanisms including osmotic adjustment that is usually accomplished by accumulation of compatible solutes such as proline, glycine betaine and polyols [33]. It has been also reported that proline levels increased in response to water stress in tomato calli [34].Taking into account the generated knowledge about plants responses to abiotic stress conditions, the determination of antioxidant enzyme activities, and levels of malonyldialdehyde and proline in plants recovered under selective conditions may help to isolated the most tolerant genotypes.

In recent years, both basic and applied research has led to understand the mechanisms underlying the stress response and the identification of the specific genes/metabolites that are responsible for tolerance phenotypes the "omics" approaches have had a significant development. Through the application of transcriptomics hundreds of genes have been linked with environmental stress responses and regulatory networks of gene expression have been delineated [35]. Moreover, plant tolerance to abiotic stress conditions has been associated with changes in proteome composition. Since proteins are directly involved in plant stress response, proteomics studies can significantly contribute to unravel the possible relationships between protein abundance and plant stress acclimation [36].

Relatively less is known about changes at the metabolomic level. Metabolome analysis has become a valuable tool to study plant metabolic changes that occur in response to abiotic stresses. This approach has already enabled to identify different compounds whose accumulation is affected by exposure to stress conditions. However, much work is still required to identify novel metabolites and pathways not yet linked to stress response and tolerance [37].

In this context, an integrated approach incorporating *in vitro* plant tissue culture to proteomics and metabolomics technique, can contribute to elucidate the metabolites involved in stress response and select desired genotypes at early stages of plant development or at callus stage [38].

5. Transgenesis for abiotic stress tolerance

Transgenic approaches are among the available tools for plant improvement programs based on biotechnological methodologies. Nowadays, many mechanisms and gene families, which confer improved productivity and adaptation to abiotic stresses, are known. These gene families can be manipulated into novel combinations, expressed ectopically, or transferred to species in which they do not naturally occur. Therefore, the possibility to transform the major crop species with genes from any biological source (plant, animal, microbial) is an extremely powerful tool for molecular plant breeding [39].

To date, successes in genetic improvement of environmental stress resistance have involved manipulation of a single or a few genes involved in signaling/regulatory pathways or that encode enzymes involved in these pathways (such as osmolytes/compatible solutes, antioxidants, molecular chaperones/osmoprotectants, and water and ion transporters [8]. The disadvantage of this approach is that there are numerous interacting genes involved, and efforts to improve crop drought tolerance through manipulation of one or a few of them is often associated with other, often undesirable, pleiotropic and phenotypic alterations [8].

The plant hormone abscisic acid (ABA) regulates the adaptive response of plants to environmental stresses such as drought, salinity, and chilling via diverse physiological and developmental processes [40, 41].

The ABA biosynthetic pathway has been deeply studied and many of the key enzymes involved in ABA synthesis have been used in transgenic plants in relation to improving abiotic stress tolerance [42]. Transgenic plants overexpressing the genes involved in ABA synthesis showed increased tolerance to drought and salinity stress [42, 43]. Similarly, many studies have illustrated the potential of manipulating CBF/DREB genes to confer improved drought tolerance [44, 45].

Another mechanism involved in plant protection to osmotic stress associated to drought and salinity involves the upregulation of compatible solutes that function primarily to maintain cell turgor, but are also involved in avoiding oxidative damage and chaperoning through direct stabilization of membranes and/or proteins [46; 47]. Many genes involved in the synthesis of these osmoprotectants have been explored for their potential in engineering plant abiotic stress tolerance [10, 47].

The cellular and metabolic processes involved in salt stress are similar to those occurring in drought-affected plants and are responses to the osmotic effect of salt [48, 49]. As described above, the use of genes related to osmoprotectant synthesis has been successfully used in developing drought-tolerant crops and the transfer of glycine betaine intermediates have improved the drought and salt tolerance of transgenic plants in many cases [50].

The amino acid proline is known to occur widely in higher plants and normally accumulates in large quantities in response to environmental stresses [51, 52]. The osmoprotectant role of proline has been verified in some crops by overexpressing genes involved in proline synthesis [53].

Other approaches successfully developed in a variety of crops to obtain abiotic-stress-tolerant plants by transgenesis, have been manipulation of transcription factors (TFs), late embryogenesis abundant (LEA) proteins, and antioxidant proteins [54].On the other hand, the use of genetic and genomic analysis to identify DNA molecular markers associated to stress resistance can facilitate breeding strategies for crop improvement. This approach is particularly useful when targeting characters controlled by several genes, as in the case of most abiotic stress.

The potential to map different Quantitative Trait Loci (QTL) contributing to an agronomical trait and to identify linked molecular markers opens up the possibility to transfer

simultaneously several QTLs and to pyramid QTLs for several agronomical traits in one improved cultivar [55]. However, the application of molecular markers in breeding programs requires preliminary studies to identify and validate potential markers [55].

Although the use of Marker-Assisted Selection may be helpful for crop improvement, its practical application in genetic improvement of resistance or tolerance to stress has been limited since no many stress tolerance QTL have been identified [56]. For future biotechnology improvements such as tolerance to drought or nutrient limitation, forward breeding will be necessary to co-optimize transgenic expression and genetic background because endogenous genes and environmental factors may have the potential to influence the phenotypes resulting from transgenic modifications [57].

It is important to point that genetic modification of higher plants by introducing DNA into their cells is a highly complex process. Practically any plant transformation experiment relies at some point on cell and tissue culture. Although the development transformation methods that avoid plant tissue culture have been described for *Arabidopsis*, and have been extended to a few crops, the ability to regenerate plants from isolated cells or tissues *in vitro* is needed for most plant transformation systems. Not all plant tissue is suited to every plant

Plant species	**stress**	**References**
Ipomoea batatas (sweet potato)	salt	[16]
Triticum aestivum (wheat)	salt	[80]
Oryza sativa (rice)	salt/drought	[81]
Lactuca sativa (lettuce)	drought/cold	[82]
Brassica juncea (mustard)	Cd	[83]
Medicago sativa (alfalfa)	freezing	[84]
Gossypium hirsutum (cotton)	chilling	[85]
Solanum tuberosum (potato)	salt-drought	[86]
Avena sativa (oat)	osmotic	[87]
Daucus carota (carrot)	salt	[88]
Zea mays (maize)	drought	[89]
Pinus taeda (loblolly pine)	salt	[90]
Populus tomentosa (chinese white poplar)	salt	[91]
Citrus sinensis x Poncirus trifoliate (Carrizo citrange)	drought	[92]
Petunia sp. (petunia)	drought	[93]
Nicotiana tabacum (tobacco)	freezing/Al	[94]
Brassica juncea (indian mustard)	As and Cd	[95]
Brassica napus (rape)	freezing	[96]
Pinus virginiana (Virginia pine)	metal	[90]
Hordeum vulgare (barley)	Al	[97]
Alyssum sp. (alyssum)	Ni	[98]

Table 2. Genetic transformation for increased resistance to abiotic stresses.

transformation method, and not all plant species can be regenerated by every method [58]. There is, therefore, a need to find both a suitable plant tissue culture/regeneration regime and a compatible plant transformation methodology. Today, many agronomical and horticultural important species are routinely transformed, and the list of species that is susceptible to *Agrobacterium*-mediated transformation seems to grow daily (Table 2).

6. *In vitro* tissue culture as a tool for physiological and biochemical studies in plants

Because of the great interest for both basic and applied research, many scientific endeavours have long addressed the understanding of the mechanisms underlying the stress response and the identification of the specific genes/metabolites that are responsible for tolerance phenotypes.

In the last decades, *in vitro* culture of plants has become an integral part of advances in plant science research. Plant tissue culture techniques allow for close monitoring and precise manipulation of plant growth and development, indeed, the *in vitro* system offers the advantage that relatively little space is needed to culture plants and this system allows a rigorous control of physical environment and nutrient status parameters, which are difficult to regulate with traditional experimental system [59]. Furthermore, any complex organ-organ and plant-environment interaction can be controlled or removed, and the level of stress can be accurately and conveniently controlled [60]. All this together makes that some aspects of plant growth, that were barely understood before the advancement of the science of tissue culture, such as the metabolism and interaction of plant hormones, as well as their physiological effects can be deeply studied [61].

Shoot apex culture has been widely used to evaluate plant physiological responses to salinity and osmotic stress in various species, including apple [59], olive [62] and tomato [63]. With regard to the whole plant, a similar response to salt stress could be expected in plantlets grown through *in vitro* shoot apex culture [63], because such explants can be considered mini-replicas of a plant with anatomical organization and ability to root and grow into whole plant.

We have previously described the use of an *in vitro* tissue culture technique to study the performance of different citrus genotypes cultured under salt stress conditions, avoiding the effect of the root by culturing shoots without the root system. The method proved to be a good tool for studying biochemical processes involved in the response of citrus to salt stress [64]. Some citrus genotypes have been classified as relatively salt tolerant under field conditions due to their ability to restrict chloride ions to roots while others have proved to be more sensitive to salinity [65].

In vitro tissue culture approach allowed us to observe that when shoots are cultured without a root system, all genotypes accumulated the same chloride levels and exhibited similar leaf damage as a consequence of salt stress treatment. There was no increase of malonyldialdehyde

levels in any genotype, and common patterns of hormonal signaling were observed among genotypes. On the view of these results we concluded that under the same salt conditions and with the same level of leaf chloride intoxication, no biochemical differences exist among tolerant and sensitive genotypes. This points to the roots as a key organ not only as a filter of chloride ions but also as a signalling system in citrus [64]. *In vitro* tissue culture provided the tools to perform this studies that it would be impossible to carry out with whole plants grown under field or greenhouse conditions.

7. Conclusion

Use of *in vitro* cell and tissue-based systems offers a remarkable tool for dissecting the physiological, biochemical and molecular regulation of plant development and stress response phenomena. In recent years, considerable progress has been made regarding the development and isolation of stress tolerant genotypes by using *in vitro* techniques.

The most successful applied tools have been the induction of somaclonal variation and *in vitro* selection of plants tolerant to different abiotic stresses and the development of transgenic genotypes throughout different approaches.

In vitro selection makes possible to save the time required for developing disease resistant and abiotic stress tolerant lines of commercial crops and other plant species. However, i*n vitro* selected variants should be finally field-tested to confirm the genetic stability of the selected traits under field conditions. The development of *in vitro* selection technology, together with molecular approaches and functional genomics will provide a new opportunity to improve stress tolerance in plants relevant to food production and environmental sustainability.

Development of transgenic plants using biotechnological tools has become important in plant-stress biology. Previous works on genetics and molecular approaches have shown that most of the abiotic stress tolerant traits are multigenic. Therefore, to improve stress tolerance several stress related genes need to be transfered. More recently manipulation of single transcription factors has provide the same effect as manipulation of multiple genes. This has become a promising approach to get abiotic stress tolerant crops.

A limiting factor for the widespread application of this technology is that, with few exceptions, genetic transformation protocols require plant regeneration of transformants using *in vitro* plant tissue culture tools. Although the list of species that are susceptible to *Agrobacterium*-mediated transformation has been increased recently, still are many genotypes for which regeneration protocols are not available.

On the other hand, plant tissue culture is also an invaluable laboratory tool to study basic aspects of plant growth and development, and to manipulate these processes since it makes possible to have a large number of plants in a small space, without the interference of other biotic or abiotic stress factors. It also allows growing plants in the same nutritional and environmental conditions all year around.

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