# In Vitro Screening of Crop Plants for Abiotic Stress Tolerance

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## 1 Introduction

Genetic variation is induced commonly through the plant tissue cultures. Some of these variations expressed as phenotypic and cytogenetical modifications in plants regenerated from the callus tissue (Bayliss 1980; Lee and PhilIips 1988). This type of variation called as somaclonal variation by Larkin and Scowcroft (1981). Somaclonal variation has been reported by many researchers and accepted as a real phenomenon among the plantlets regenerated from callus. Most likely, somaclonal variations are originated through the exposure of dedifferentiated tissues to culture cycles (Remotti 1998) or transferring some or all cells from a previous culture to fresh growth medium (subcultures), the intensity of the somaclonal variations vary through the genotype and the genetic base of the species (Karp 1995) and tissue culture conditions. Cell and tissue culture conditions may minimize or maximize the extent of somaclonal variations.

Over the years, many variations in the form of mutations in the genomes of plants have been naturally evolved. Plant breeders took a great advantage of these

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variations for breeding purposes. Thus, wild plants have become a valuable source of variations for plant breeders. However, these variations are limited and their production rate is very low. Thus, plant and crop breeders enhanced the chance of variations using chemicals, ionized radiations and ion beams (Meksem and Kahl 2009). Chemicals such as alkylating agents (e.g., Ethyl methanesulfonate (EMS) produces random mutations in the genome by nucleotide substitution, particularly by nucleotide alkylation. Ionized radiations of neutrons and gamma rays are useful for creation of more changes in the genome. Ion beams such as carbon ions omit nucleotide substitution which could be useful for plant breeding programs. However ionized radiation and ion beams need advanced equipment, unfortunately many laboratories are poorly equipped. Chemicals such as EMS, are usually used in some laboratory to create a variation in the plant genome, however its toxic effects are strongly dangerous for operator. However, creation of somaclonal variations can be appropriate way for acquisition of genetic diversity among crops. This type of variations has many advantages: (1) plant tissue culture creates the mutation in the regenerated plants, (2) materials used in tissue culture media are not toxic for the user such as chemicals (EMS), (3) to create somaclonal variations advanced equipment are not necessary, (4) it is a simple process to create somaclonal variation in vitro in any laboratory, (5) plant breeders can steer the somaclonal variations toward creation of desired traits in in vitro-regenerated plants by using special agents. Random variations in the plant genome are caused due to the use of chemicals, ionized radiation and ion beams whereas, in vitro screening methods can modify a specific trait in plants. Due to the widespread occurrence of abiotic stresses because of climate change. Need arises for plant breeders to improve tolerance of crops to environmental stresses using conventional breeding and gene transformation methods. In the conventional breeding method, much time should be spent creating tolerant progeny against various stresses. In addition, a large volume of field-work should be spent to plant a progeny and elected tolerant progenies to environmental stresses. In in vitro selecting method, potential tolerant progeny screened using special agents. In fact, compounds in tissue culture medium are selected to grow only tolerant offspring. Thus, for plant breeders, this method is much economic and faster compared to conventional methods. By screening, regenerated plantlets will be assessed for their tolerance to environmental stresses in the greenhouse and farm respectively.

## 2 Screening for Various Abiotic Stress Tolerance

### *2.1 Cold Stress*

Improvement of frost tolerance in winter cereals using conventional breeding has been a slow process which is possibly due to ‘limited genetic variation in the gene pools’ (Limin and Fowler 1993). Molecular methods for this trait have not shown any significant results in winter cereals due to its polygenic control.

As an alternative way to improve cold hardiness of winter barley, a biotechnological approach based on somaclonal variation in tissue culture can be used (Tantau et al. 2004a, b). Different studies have shown the accumulation of proline during cold hardening in many plants including cereals (Dorffling et al. 1993). The level of proline correlates positively with genotype specific frost tolerance in these crops (Tantau et al. 2004a, b). Moreover, proline has protective functions in different plants under abiotic stress such as osmotic (Delanauney and Verma 1993), salinity (Nanjo et al. 1999) and frost stress (Nanjo et al. 1999). Therefore, many researchers believed that any procedure that increases the level of proline in plants should result in an increase in frost tolerance (Tantau et al. 2004a, b).

Successful efforts have been made to increase proline through manipulation of the proline biosynthesis or degrading system by gene engineering to improve stress tolerances. Improvement of salinity tolerance in tobacco, wheat, strawberry, *Brassica napus* and sorghum plants was obtained by overexpression of the key enzyme of proline synthesis, Δ-1-pyrroline-5-carboxylate synthetase, 'P5CS' (Ahmed et al. 2015; Hong et al. 2000; Sawahel and Hassan 2002; Bahramnejad et al. 2015; Kubala et al. 2015; Reddy et al. 2015). Nanjo et al. (1999) improved freezing and salinity tolerance in Arabidopsis by antisense suppression of proline degradation which resulted in overaccumulation of proline.

Besides genetic engineering techniques, based on somaclonal variation and biochemical markers as selection tools, in vitro culture can be used to modify crops for abiotic stress tolerance. In the case of frost tolerance, many studies showed the positive correlation between frost tolerance and proline accumulation. In fact, conventional breeding programs have used proline accumulation as a biochemical marker for increased frost tolerance in some crops (Winkel 1989). Thus, selection of high-proline genotypes may yield improved frost tolerance which can be the first way for screening frost tolerance calli. Van Swaaij et al. (1986, 1987) were the first researchers to increased frost tolerance in potato using hydroxyproline (Hyp), through the in vitro selection. To obtain frost tolerance, embryogenic calli should expose to Hyp as a selection agent to select cell lines with an increased level of proline (Fig. 1). They are assumed to be able to overcome the toxic effect of Hyp. plants will regenerate from selected cell lines and frost tolerance and winter survival in the field are determined in the regenerated plants and their progenies as well as proline levels in the progenies. Crossing experiments can be carried out to confirm the heritability of the trait 'increased frost tolerance' (Tantau et al. 2004a, b).

Many researchers tried to examine HYP as selective agents for screening of cell lines with an increased level of proline (Table 1). Tantau et al. (2004a, b) were plated embryogenic calli derived from anther cultures of the two-rowed winter barley cultivar 'Igri' on solid L3 medium containing the proline analogue hydroxyproline (Hyp), 10–20 mmol/L. A sever degeneration was observed in most calli in the presence of HYP. Hyp resistant calli were distinguished by their lighter color and higher growth rate. From 22,500 anthers exposed to Hyp, 46 Hyp resistant regenerates were selected and then transferred to soil. After 5–10 weeks cultivation at normal growth conditions, they were cold hardened at 2 °C under short day conditions together with control regenerates. Frost tolerance assays revealed that Hyp resistant

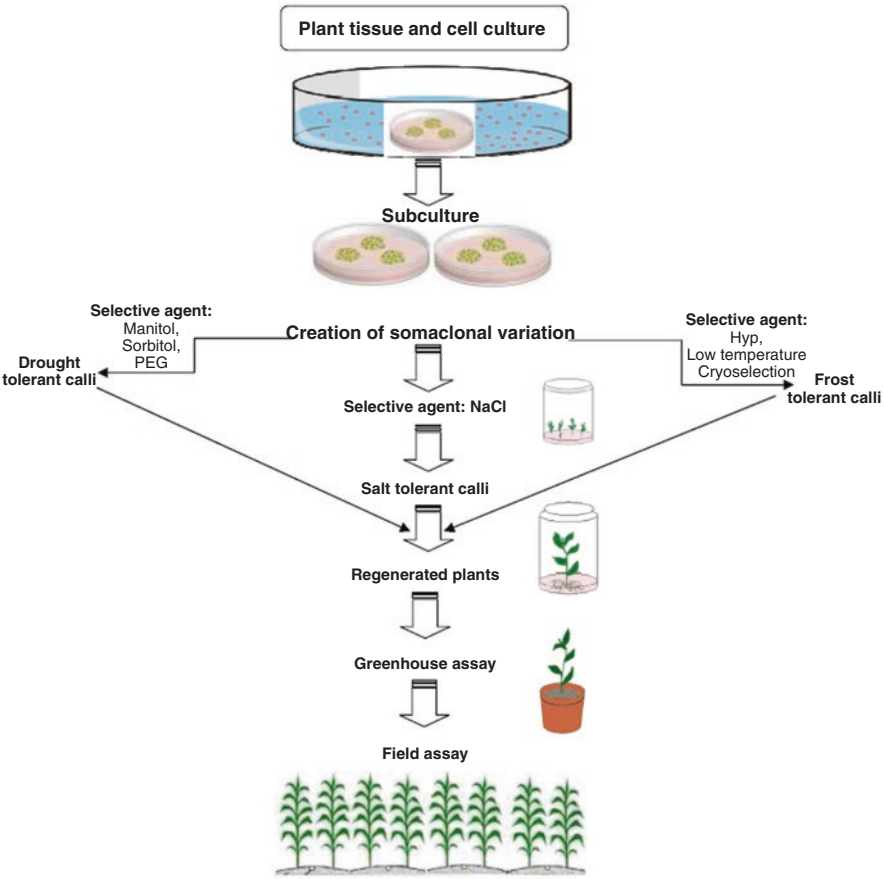


**Fig. 1** A schematic diagram illustrating the procedure for development of abiotic stress tolerance in plants

**Table 1** List of some plant species showing tolerance to various abiotic stresses through in vitro selection

| Plant species | Type of stress | Selecting agents | References |
|---|---|---|---|
| *Triticum aestivum* | Cold stress | – | Lazar et al. (1988) |
| *Triticum aestivum* | Cold stress | Cryoselection (immersion in liquid nitrogen without addition of cryoprotectants) | Kendall et al. (1990) |
| *Triticum aestivum* | Cold stress | Hydroxyproline | Tantau and Dörffling (1991) |
| *Triticum aestivum* | Cold stress | Hydroxyproline | Dorffling et al. (1993) |
| *Triticum aestivum* | Salt stress | NaCl | Karadimova and Djambova (1993) |
| *Triticum aestivum* | Cold stress | Hydroxyproline | Dörffling et al. (1997) |

(continued)

**Table 1** (continued)

| Plant species | Type of stress | Selecting agents | References |
|---|---|---|---|
| *Oryza sativa* | Cold stress | Different temperature regimes | Bertin and Bouharmont (1997) |
| *Solanum tuberosum* | Cold stress | Hydroxyproline | Anjum (1998) |
| *Paspalum vaginatum* | Cold stress | Different temperature regimes | Liu et al. (2013) |
| *Hordeum vulgare* | Cold stress | Hydroxyproline | Tantau et al. (2004a, b) |
| *Oryza sativa* | Drought stress | PEG | Biswas et al. (2002) |
| *Solanum lycopersicum* | Drought stress | PEG | Aazami et al. (2010) |
| *Oryza sativa* | Drought stress | PEG | Wani et al. (2010) |
| *Oryza sativa* | Drought stress | PEG | Joshi et al. (2011) |
| *Solanum tuberosum* | Drought stress | Sorbitol | Albiski et al. (2012) |
| *Triticum aestivum* | Drought stress | PEG | Mahmood et al. (2012) |
| *Pennisetum ciliare* | Drought stress | Mannitol | Carloni et al. (2017) |
| *Brassica juncea* | Drought stress | Mannitol | Gangopadhyay et al. (1997) |
| *Capsicum annuum* | Drought stress | PEG | Santos-Diaz and Ochoa-Alejo (1994) |
| *Daucus carota* | Drought stress | PEG | Fallon and Phillips (1989) |
| *Saccharum sp.* | Drought stress | Mannitol | Errabii et al. (2006) |
| *Solanum tuberosum* | Drought stress | Mannitol | Sabbah and Tal (1990) |
| *Sorghum bicolor* | Drought stress | PEG | Smith et al. (1985) |
| *Sorghum bicolor* | Drought stress | PEG | Duncan et al. (1995) |
| *Triticum aestivum* | Drought stress | PEG | Dorffling et al. (1993) |
| *Triticum aestivum* | Drought stress | PEG | Barakat and Abdel-Latif (1996) |
| *Triticum aestivum* | Drought stress | PEG | Barakat and Abdel-Latif (1995) |
| *Triticum aestivum* | Drought stress | PEG | El-Haris and Barakat (1998) |
| *Triticum durum* | Drought stress | PEG | Hasissou and Bouharmont (1994) |
| *Solanum tuberosum* | Salt stress | NaCl | Sajid and Aftab (2014) |
| *Maniho tesculenta* | Salt stress | NaCl | El-Minisy et al. (2016) |
| *Brassica juncea* | Salt stress | NaCl | Jain et al. (1990) |
| *Brassica oleracea* | Salt stress | NaCl | Elavumoottil et al. (2003) |
| *Brassica napus* | Salt stress | NaCl | Rahman et al. (1995) |
| *Chrysanthemum morifolium* | Salt stress | NaCl | Hossain et al. (2007) |
| *Diplachnefusca* | Salt stress | NaCl | Nanakorn et al. (2003) |
| *Fragaria×ananassa* | Salt stress | NaCl | Dziadczyk et al. (2003) |
| *Glycine max* | Salt stress | NaCl | Liu and Staden (2000) |
| *Hordeum vulgare* | Salt stress | NaCl | Ye et al. (1987) |

(continued)

**Table 1** (continued)

| Plant species | Type of stress | Selecting agents | References |
|---|---|---|---|
| *Helianthus annus* | Salt stress | NaCl | Davenport et al. (2003) |
| *Ipomoea batatas* | Salt stress | NaCl | He et al. (2009) |
| *Linum usitatissimum* | Salt stress | NaCl | McHughen (1987) |
| *Lycopersiconesculentum* | Salt stress | NaCl | Kripkyy et al. (2001) |
| *Lycopersicon peruvianum* | Salt stress | NaCl | Hassan and Wilkins (1988) |
| *Medicago sativa* | Salt stress | NaCl | McCoy (1987) |
| *Medicago sativa* | Salt stress | NaCl | Safarnejad et al. (1996) |
| *Nicotiana tabacum* | Salt stress | NaCl | Rout et al. (2008) |
| *Oryza sativa* | Salt stress | NaCl | Binh and Heszky (1990), |
| *Oryza sativa* | Salt stress | NaCl | Basu et al. (1997) |
| *Oryza sativa* | Salt stress | NaCl | Shankhdhar et al. (2000) |
| *Oryza sativa* | Salt stress | NaCl | Lee et al. (2003) |
| *Saccharum sp.* | Salt stress | NaCl | Gandonou et al. (2006) |
| *Solanum tuberosum* | Salt stress | NaCl | Sabbah and Tal (1990) |
| *Solanum tuberosum* | Salt stress | NaCl | Ochatt et al. (1999) |
| *Solanum tuberosum* | Salt stress | NaCl | Queiros et al. (2007) |
| *Triticum aestivum* | Salt stress | NaCl | Vajrabhaya et al. (1989) |
| *Triticum aestivum* | Salt stress | NaCl | Karadimova and Djambova (1993) |
| *Triticum aestivum* | Salt stress | NaCl | Barakat and Abdel-Latif (1996) |
| *Triticum aestivum* | Salt stress | NaCl | Zair et al. (2003) |
| *Vigna radiata* | Salt stress | NaCl | Hassan et al. (2008) |

regenerants were significantly more frost tolerant than the control regenerants. Improved frost tolerance was found also in the progenies R1 to R9, and genotypic segregation in the R1 generation in a 1:2:1 ratio was indicated. A significant increase in proline content was observed in the R2 generation and in subsequent generations ($P \leq 0.001$) which was correlated with increased frost tolerance in the Hyp lines. The results support the hypothesis that proline accumulation in cold acclimated winter barley plants is related to the acquisition of frost tolerance. Moreover, the described biotechnological procedure may be applicable in breeding programs for improved winter hardiness and possibly also for other stress tolerances.

Five hundred hydroxyproline-resistant cell lines were selected from cell cultures of wheat (*Triticum aestivum* L. cv. Koga II) after plating on 10–30 mM hydroxyproline (Hyp) containing solid Gamborg B 5 medium (Tantau and Dorffling 1991). All selected cell lines from 30 mAf Hyp-medium contained increased (up to 17-fold) levels of free proline. Seventy-four cell lines were transferred to Hyp-free medium and sub-cultivated 25 times for 12 months altogether, until 80% still were showing increased proline levels. Fourteen cell lines with increased proline levels were further investigated in liquid media based on their frost tolerance, which was measured by

means of electrolyte leakage. Ten of them showed increased frost tolerance with LT 50 values as low as 2.7 °C below that of the wild type (4.7 °C). Besides increased proline levels and increased percentage of dry weight, the Hyp-resistant cell lines had lower osmotic potentials. Osmotic potentials correlated better than levels of free proline with the increase in frost tolerance.

Dorffling et al. (1993) used immature embryos of a Finnish winter wheat (*Triticum aestivum* L. cv. Jo 3063) for in vitro-selection of hydroxyproline (Hyp) resistant calli plated on solid Gamborg B5 medium containing 10–20 mM Hyp and 2 mg/L 2,4-D (Dorffling et al. 1993). From 6018 embryogenic calli exposed to Hyp in the course of three subcultures, 9 calli proved to be Hyp-resistant and remained viable and embryogenic. The regenerated plants were grown at 18 °C for 6 weeks and then cold hardened at 2 °C for 18 weeks. Their results showed that the mean osmotic potential of the Hyp-resistant cold hardened regenerates was significantly lower than that of hardened controls. At the same time their mean proline content and their mean frost tolerance were significantly higher compared with regenerated controls.

As mentioned above, Dorffling et al. (1993) reported in vitro-selection of proline over accumulating lines of winter wheat (*Triticum sativum* L. cv. Jo 3063) with increased frost tolerance. Then, the improvement of frost tolerance (winter hardiness) under field conditions is confirmed for $F_7$ progenies of the mutants (Dorffling et al. 2009). Moreover, the mutants accumulated higher levels of glucose, fructose, soluble protein and abscisic acid (ABA) in addition to proline compared to the wild type. This can occur under cold hardening conditions either in growth chamber or field conditions. ABA and proline levels reached to peak when the temperature dropped, whereas carbohydrate levels slowly increased at decreasing temperature. Soluble protein levels also increased during cold hardening, however this showed a sharp decline during frost periods. Increased carbohydrate levels of the mutants were associated with lower osmotic potential values. The differences in carbohydrate, protein and ABA levels between the mutants and the wild type are probably due to pleiotropic effects of the mutation.

In addition to the use of HYP containing medium for selection of frost tolerance calli, some researchers used just low temperature for distinguish of frost tolerance calli. For example, Liu et al. (2013) improved cold tolerance in warm season turf grass species using in vitro selection. Embryogenic calli were subjected to 2 or 6 °C treatment for 90 days for in vitro cold selection of somaclonal variation. Plants regenerated from calli surviving cold treatment (cold-selected) after 45 or 60 days were then exposed to low temperatures [15/10 or 5/3 8 °C (day/night)]. Plant variants derived from cold-selected calli exhibited a significant improvement in their tolerance to low temperature of either 15/10 or 5/3 8 °C (day/night), as manifested by higher turf quality, leaf chlorophyll content, and membrane stability as well as lower levels of lipid peroxidation compared with the control plants. This study demonstrated the feasibility of in vitro selection for cold tolerance in seashore paspalum.

In another study, progeny of 66 plants regenerated from callus cultures derived from immature embryos of Norstar winter wheat were evaluated as seedlings for tolerance to controlled freezing (Lazar et al. 1988). Greater freezing tolerance compared the parent cultivar was observed in both R2 and R3 regenerated families.

LT50 values (predicted temperatures at which mean survival frequencies are 50%) for four families in the R2 generation and three families in the R3 were significantly lower than that of Norstar.

Embryo-derived calli of four rice varieties cultivated at high altitude in Burundi-Facagro 57, Facagro 76, Kirundo 3 and Kirundo 9 were submitted to different temperature regimes (Bertin and Bouharmont 1997). The percentage of regenerating calli greatly varied depending on variety, length of culture and callus temperature treatment. The reduction of regeneration percentages induced by low temperature which was more pronounced in the more sensitive varieties. Regenerated plants (R0) and their progenies in R1, R2 and R3 were cold-screened together with control plants. In all varieties, significantly higher survival rates were obtained in R3 with in vitro plants than with control plants. Such chilling tolerance improvement was not obtained following a massal selection applied during three successive generations onto the control plants. In vitro plants regenerated from calli cultivated either at 25 °C, or at 4 °C, were cultivated at different altitudes in Burundi during two successive generations. For most observed traits, the in vitro plants were characterized by lower means, larger variation and higher maximum values than the control plants. The most chilling-tolerant somaclonal families were most usually characterized by extensive differences in fatty acid composition, chilling-induced electrolyte leakage and chlorophyll fluorescence, compared to the varieties which were derived from.

Kendall et al. (1990) developed a cryoselection protocol that provides freezing-tolerant callus that, in turn, can regenerate plants with enhanced cold hardiness (Kendall et al. 1990). Tolerant calli were selected from spring wheat (*Triticum aestivum* L.) callus by immersion in liquid nitrogen without addition of cryoprotectants. Less than 15% of the calli survived the initial challenge, whereas 30–40% of previously selected calli survived subsequent exposure. Seed progeny from 5 of 11 regenerant (R2) lines tested exhibited significantly enhanced tolerance to freezing at –12 °C. Thus, cryoselection appears to involve at least in part, selection for genetic rather than epigenetic variants. Analysis of one callus line indicated that cryoselection did not induce significant alterations in lipid composition, adenylate energy charge or freezing point. An increase in the soluble sugar component was detected. Changes were also detected in the protein complement of microsomal membrane and soluble protein extracts of cryoselected callus. In all, seven unique proteins ranging from 79 to 149 kDa were identified. The results demonstrated that freezing tolerant callus can be isolated from a heterogeneous population by cryoselection, and factors that contribute to hardiness at the callus level which are biologically stable and can contribute to tolerance at the whole plant level.

## *2.2 Drought Stress*

Drought in agriculture is defined as inadequacy of water availability, including precipitation and soil-moisture storage capacity in quantity and distribution during the life cycle of a crop plant. This restricts the expression of full genetic potential of

the plant (Sinha 1986). Drought is one of the most important environmental stresses that occur in different parts of the world and act as a major limiting factor to prevent the maximum crop yield (Mitra 2001).

Improving drought tolerance and productivity is one of the most difficult tasks for cereal breeders. The difficulty arises from the diverse strategies adopted by plants themselves to combat drought stress depending on the timing, severity and stage of crop growth. Compounding the problem further are the many loci that show efficacy only in a subset of circumstances (Tuinstra et al. 1996; Nguyen et al. 2004).

Breeding of drought tolerance by conventional methods seems to be difficult because the yield heritability is critically low under drought condition due to small genotypic variance or large genotype-environment interaction variances (Blum 1988). The genetic structure and phenotypic expression of a quantitative trait are highly influenced by environmental factors. Thus, one barrier for understanding of inheritance in a quantitative trait is genotype-environment interactions (Breese 1969).

Tissue culture introduces the special methods for selecting individuals in in vitro condition by adding selective agents to the culture medium (Mohamed et al. 2000; Lu et al. 2007), either directly or gradually (Gangopadhyay et al. 1997; Hassan et al. 2004; Mohamed and Ibrahim 2012). The base of this method is creating genetic variations during cell or tissue culture and then recovers of individuals (Biswas et al. 2002; Matheka et al. 2008; Lu et al. 2009; Verma et al. 2013). The agent has been applied at different growth stages: during the callus induction process, during seedling regeneration or all throughout the in vitro culture stages (Biswas et al. 2002; Errabii et al. 2007; Aazami et al. 2010; Verma et al. 2013). Drought stress conditions are usually simulated by adding compounds, such as mannitol, sorbitol or polyethylene glycol (PEG) (Fig. 1) (Leone et al. 1994; Joshi et al. 2011; Mahmood et al. 2012), which reduce the water potential of the medium. The responses of the different explants or in vitro regenerated plants can be influenced by secondary effects, either morphological or physiological, of the compounds used to simulate stress (Hohl and Schopfer 1991; Verslues et al. 1998; Cha-um et al. 2012). For this reason, besides confirming the efficiency of in vitro selection, all selection processes should include an ex vitro assay to determine the exact measure of tolerance to the osmotic agent observed in the laboratory (Remotti 1998).

Many researchers used somaclonal variations to improve drought tolerance of crops using selective agents (Table 1). Carloni et al. (2017) tried to define a protocol for in vitro selection of drought tolerant calli of buffelgrass using mannitol. Buffelgrass is a forage grass that reproduces mainly by apomixis. In species with this reproduction mode, in vitro selection allows the incorporation of alternatives in a breeding program (Carloni et al. 2017). In the embryogenic callus induction medium (IM), the highest values of the variables fresh weight of embryogenic calli, proportion of embryogenic calli and number of regenerated seedlings (NRS) were obtained in the 25 mM mannitol treatment. The remaining concentrations of the osmotic agent (50, 75, 100 and 150 mM) had a negative effect on these variables. In the regeneration medium (RM), NRS was reduced at all mannitol concentrations. When embryogenic calli were induced and seedlings were regenerated maintaining mannitol concentrations in IM and RM, the highest NRS values were recorded at

25 mM mannitol. In vitro regenerated seedlings transplanted to an experimental plot exhibited different morphological characteristics from those of the anther donor plant. ISSR primers detected 22% of polymorphic bands and divergence between 0.20 and 0.37 in in vitro regenerated plants. Finally, water stress assays confirmed that S1 progenies exhibited a differential behavior from that of the parent material. Under 100 mM of mannitol used as selection pressure in IM or in both IM and RM, S1 progenies of two regenerated materials had higher height, fresh weight and dry weight at the end of water stress assay.

### 2.2.1 Salt Stress

Salt is called as high concentration of soluble salts in the soil. Soil with 4 dS/m ECe or more is approximately equal to 40 mM NaCl and 2/0 MPa osmotic pressure is considered as salty soils. This definition is taken from the ECe that significantly affect crop yield more. NaCl is most abundant salt solution and all plants employ mechanisms to adjust its accumulation (Munns and Tester 2008).

More than 800 million hectares of land worldwide are affected by salinity (Munns and Tester 2008). This amount is more than 6% of the world's land. Most of the land affected by salinity caused by natural factors. This is the result of long-term accumulation of salts during the period when the lands are arid and semiarid (Rengasamy 2002). Apart from the natural salt, an important part of agricultural land that planted recently are salty due to changes in land use for agricultural purposes. Both factors increase the concentration of salts in the root area (Munns and Tester 2008).

Plants vary widely in salt tolerance. Among the cereals, rice (*Oryza sativa*) is the most sensitive and barley (*Hordeum vulgare*) is the most tolerant to salinity. Bread wheat (*Triticum aestivum*) have average tolerance and durum wheat (*Triticum turgidum* ssp durum) are less tolerant to salinity (Munns and tester 2008). Salt tolerance in dicotyledonous have more diversity (Munns and Tester 2008).

**Salinity affects the plants in two ways:**

1. Osmotic phase, a high concentration of salts in the soil, makes it difficult to uptake the water by root (Munns and Tester 2008). Salts out of the root can have a fast effect on cell growth and the related metabolism. This cause a reduction in leaves growth, new leaves and lateral buds develop slowly so, fewer side branches form (Munns and Tester 2008). The main effect of salinity on barley will emerge as a decrease in the number of tillers. In dicotyledonous species, the main effect of salinity significantly reduces the size of individual leaves or branches (Munns and Tester 2008).
2. Ion-specific phase, salts gradually accumulate within the plant to reach toxic concentration (Munns and tester 2008). This can be started with the accumulation of salt in toxic concentrations in the old leaves. This leaves have less efficient detoxification of salts and eventually die. If the mortality rate of the old leaves become greater than the younger leaves, the growth rate decreases further

(Munns and Tester 2008). Ion stress overcomes the osmotic effect of the ion just in high salinity levels or sensitive species that lack the ability to control the transfer of sodium (Munns and Tester 2008).

Increased salinity is one of the major constraints on crop productivity. Necessity of plant production with increased salt tolerance has been extensively emphasized by increased crop research (Munns et al. 2002; Flowers 2004). In vitro culture techniques are an excellent tool to study the behavior of undifferentiated cells and the bulk plants in ambient stress under control conditions (Sajid and Aftab 2014). The exploitation of somaclonal variation is also potentially quite useful for in vitro selection of cells and tissues against several stresses (Bajaj 1987; Tal 1996). For salt tolerance, different concentration of NaCl can be used to select a salt tolerant calli. So far, different studies have shown a successful in vitro selection of $Na^+$ tolerant calli for various plants. For salt tolerance, different concentration of NaCl can be used to select a salt tolerant calli (Fig. 1). So far, different studies have shown a successful in vitro selection of $Na^+$ tolerant calli for various plants (Table 1).

Sajid and Aftab (2014) reported an in vitro direct selection of salt-tolerant callus cultures and subsequent plant regeneration in two potato cultivars (Cardinal and Desiree). Results have shown more than 50% reduction in relative fresh callus mass in the two potato cultivars exposed to 120 mM NaCl. Callus morphology correspondingly changed from off-white to blackish-brown at 120 mM to acutely-necrotic at 140 mM NaCl. Regeneration potential of recurrently-selected callus cultures (100 mM NaCl-treated) on salt-free regeneration medium (MS + 2.64 μM NAA and 1.00 μM TDZ) was not much different as compared to the control (non-selected ones). Regenerated plants from salt-tolerant callus cultures of both the cultivars after selection were transferred to soil and organic matter (50:50, v/v) for climatization in the greenhouse. It was observed that the recurrently selected plants had higher fresh/dry weight and number of tubers compared with the control ones in both cultivars. Likewise the protein, peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD) activities have shown an increasing trend in salt-treated plants of both cultivars. The results from this study highlighted a strong possibility for the selection of salt-tolerant callus lines of potato followed by an efficient plant regeneration and further acclimatization.

In another study, the calli cultures of *Guizotia abyssinica* (niger) cultivars IGP 76 and GA 10 were exposed to different levels of salt treatments (0, 30, 60, and 90 mM NaCl), in order to evaluate growth, physiological, and biochemical responses (Ghane et al. 2014). A significant decrease in relative growth rate and tissue water content of GA 10 calli than IGP 76 under salt-stress conditions was associated with higher sodium ion accumulation. Osmotic adjustment revealed by the osmolytes accumulation was significantly higher in IGP 76 salt-stressed calli as compared to GA 10. The sustained growth and better survival of IGP 76 calli was correlated with lower malondialdehyde content and increased antioxidant activities and higher α-tocopherol content in comparison to GA 10. The higher osmolytes accumulation and presence of better antioxidant system suggested superior adaptation of IGP 76 calli on salt-containing medium for prolonged periods in comparison to GA 10. The regeneration frequency, organogenesis and acclimatization response of the plants derived from

salt-adapted calli was comparatively lower than the plants derived from control calli of IGP 76. The growth, physiological and biochemical characterization of the salt-tolerant regenerated plants exposed to stepwise long-term 90 mM NaCl treatment revealed no significant changes in comparison to the control. Thus, their results suggests the development of an efficient protocol for in vitro selection and production of salt-tolerant plants in self-incompatible crop, and an alternative to traditional breeding programs to increase the abiotic stress tolerance.

In another study, Cassava suspension culture grown on MS media containing 50, 100, 150, 200 and 250 mM NaCl were established from cassava callus cultures were all dramatically induced in response to salt treatment (El-Minisy et al. 2016). The results indicated that the high NaCl concentration of 200 and 250 mM decreased one-fold the viable cell number compared to lower concentrations and control sample. Surprisingly at 50, 100 and 150 mM NaCl higher number of viable cells were found compared to control sample. However, the cell viability in 12 days of NaCl stress showed high tolerance against salt stress and the cell numbers also higher compared to other NaCl concentrations. Ionic status suggested that 200 mM NaCl accumulated less $Na^+$, $Cl^-$ and $Ca^{2+}$ and maintained better $K^+$ in comparison to other NaCl stress cell samples. The ion homeostasis data of cassava cell culture under NaCl stress showed that the $Na^+$ and $K^+$ accumulation increased very much under lower concentrations of NaCl and gradually decrease in higher concentration. There is a positive relationship between salt tolerance and proline content in *in cassava* cultures up to 200 mM NaCl stress and the highest proline content compared to other treatments. Gel activity assay of superoxide dismutase (SOD), peroxidase (GPX) and total peroxidase (POX) activity increased in tolerant cell lines as compared to control. Analysis of the above enzymes suggests that selected cassava cell lines possessed more efficient scavenging system of reactive oxygen species under 200 mL NaCl. It can be concluded that in cassava suspension culture viability of cell under 200 mM NaCl stress after 15 day will be the perfect time to isolate and identify the intercellular and extracellular protein or/and peptides which could be produced abundantly.

## 3 Conclusion

Due to the mounting food demand worldwide, plant breeders are seeking fast, low-cost and safe methods for breeding of crops especially tolerant ones against abiotic stresses. In vitro selection can be the appropriate choice for plant breeders because of its advantages mentioned in this review. In vitro selection methods rely on somaclonal variation produced due to mutations in plants regenerated from tissue culture. For creation this type of variation, there is no need for advanced equipment, time and spaces and toxic chemicals for creation of mutations. In addition, plant breeders can direct the somaclonal variations toward creation of desired traits in in vitro regenerated plants by using special agents. It seems that this method will play a more important role for breeding crops in the future.

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