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Review Article: Application of Tissue Culture on Biotic Stress Tolerance in Plants

Zeleke Keimeso*

EIAR- Ambo Agriculture Research Center, PO Box 37, Ambo, Ethiopia

**Corresponding author*

Abstract

In the face of predicted increases in the world population to around 10 billion by 2050 and the challenges faced by agriculture as a result of climate change, providing adequate food and fiber for humanity is a pressing issue requiring urgent attention. Therefore, the efforts to develop stress tolerant plants are of immense importance to increase crop productivity. In recent years, tissue culture based in vitro selection has emerged as a feasible and cost-effective tool for developing stress-tolerant plants. Plants tolerant to biotic stresses can be acquired by applying the selecting agents such as pathogen culture filtrate, phytotoxin or pathogen itself (for disease resistance) in the culture media. Only the explants capable of sustaining such environments survive in the long run and are selected. In vitro selection is based on the induction of genetic variation among cells, tissues and/or organs in cultured and regenerated plants. The selection of somaclonal variations appearing in the regenerated plants may be genetically stable and useful in crop improvement. This review focuses on the progress made towards the development of biotic stress-tolerant lines through tissue culture based in vitro selection.

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Introduction

One way to increase the quantity and quality of food is to reduce damages caused by insects, diseases and weeds to crops. Pathogens cause losses in 10-16% of the global harvest (Chakraborty *et al.*, 2011). This figure for pest damage is about 14-25% of the total agricultural production (DeVilliers *et al.*, 2011).Damages caused by these stresses are responsible for enormous economic losses worldwide. Traditional breeding technologies and proper management strategies continue to play a vital role in crop improvement. The conventional breeding programmes are being employed to integrate favorable genes of interest from inter crossing genera and species into the crops to induce stress tolerance. However, conventional breeding methods have little success and have failed to provide desirable results (Purohit *et al.*, 1998). Therefore, we need to deploy the biotechnological tools for addressing the critical problems of crop improvement for sustainable agriculture. Genetic engineering for developing stress tolerant plants, based on introgression of genes that are known to be involved in stress response and putative tolerance, might prove to be a faster track towards improving crop varieties. Genetic transformation is now a widely used procedure for introducing genes from distant genepools into many plant species for the development of stress tolerant plants and considerable efforts have been made to produce stress-tolerant plants using this technique (Borsani *et al.*, 2003; Yamaguchi and Blumwald, 2005). However, the major limiting factors in extension of this technique to several stresses are the silencing of transgene,

consequent reduction of gene expression and low transformation frequency (Mondal *et al.,* 1997). Tissue culture technique has emerged as a feasible and cost-effective alternative tool for developing stress-tolerant plants in recent years. This technique can operate under controlled conditions with limited space and time (Sakhanokho and Kelley, 2009), and has the potential for selection of stress-tolerant variants using a low cost laboratory set up.

Applications of In Vitro Techniques in Plant Breeding

Plant tissue culture technology began with Gottlieb Haberlandt's theory of cell totipotency at the beginning of twentieth century (Vasil, 2008). Following on from this, the discovery of auxins by Frits Warmolt Went in 1926 (Pennazio, 2002), and cytokinins by Folke Skoog and colleagues in the 1950s (Kieber, 2002), led to the first success of in vitro techniques in plant tissues. Since then, the technology has developed considerably and now plays a key role in genetic engineering and crop improvement. Plant tissue culture offers an array of techniques that complement conventional plant propagation and plant breeding methods. The most common reasons for the use of in vitro techniques has been for plant propagation, but its most important application in recent years has been to crop improvement using gene technology (Thakur *et al.,* 2012). Techniques such as in vitro fertilization and protoplast fusion enable the recombination of genotypes otherwise limited by incompatibility (Sri Rama Murthy *et al.,* 2012; Tapingkae *et al.,* 2012). Conventional breeding can be hastened by exploiting increased genetic diversity resulting from somatic variability (Bairu *et al.,* 2011; Nwauzoma and Jaja, 2013).

Application of Tissue Culture inSomatic Embryogenesis

The term somatic embryogenesis refers to the process of embryo development from cells other than gametes (somatic cells) without a normal fertilization process. The phenomenon of somatic embryogenesis was first reported by Steward *et al.,* (1958) on suspension culture of Daucus carota, and by Reinert (1959) on callus culture of the same species.

Somatic embryogenesis can be used in a number of ways. For example, large scale-clonal propagation of elite cultivars (Ahmad *et al.,* 2011), producing artificial seeds (synthetic seeds) (Pintos *et al.,* 2008), gene transfer for genetic improvement (Li *et al.,* 2002), in vitro selection approaches for various biotic and abiotic stresses (Ahmad *et al.,* 2011), and providing potential models for studying molecular, regulatory and morphogenetic events during plant embryogenesis (Ravi and Anand 2012). Slater *et al.,* (2003) claimed that somatic embryos may be produced indirectly by involving the dedifferentiation of organized tissue into the callus mass prior to embryo formation, or embryos may be produced directly from organized tissue without an intervening callus phase. The anatomical and physiological features of embryos derived from somatic tissues are highly comparable to zygotic embryos derived through normal fertilization (Dobrowolska *et al.,* 2012).

Application of Tissue Culture in Development of Somaclonal Variation

Characteristics for which somaclonal mutants can be improved during in vitro culture includes resistance to disease, herbicides and tolerance to environmental or chemical stress, as well as for increased production of secondary metabolites. Selection is done by employing a stress-causing agent in tissue culture containing dividing cells. An efficient method for obtaining plants with desired characteristics is to add selective agents that will alter other aspects of the phenotype Tapingkae *et al.,* (2012). Somaclonal variation has been associated with changes in chromosome numbers (polyploidy and a euploidy), chromosome structure (translocations, deletions, insertions and duplications), point mutations, and DNA methylation (Nwauzomaand Jaja, 2013; Rodriguez-Enriquez *et al.,* 2011).

The molecular basis of somaclonal variation is not precisely known; however, both genetic and epigenetic mechanisms are suggested to play a role (Jiang *et al.,* 2011).Changes in DNA methylation often give rise to epigenetic effects, which can affect expression of genes normally suppressed. Epigenetic variation is often unstable and can disappear either after plants are removed from culture or within a few clonal generations, whereas genetic variation is heritable (Biswas *et al.,* 2009). Therefore, the success in applying somaclonal variation in plant breeding is dependent on the genetic stability of the selected somaclones.

Application of Tissue Culture in Embryo Rescue

Embryo rescue is in vitro techniques aiming to encourage the development of immature embryos into complete plants. This technique has been widely used to

avoid embryo abortion in regenerated plants from hybridization. It can also be applied to shorten the breeding cycle by overcoming dormancy in seeds. The technique was first developed by Tukey in 1933 who successfully grew the embryo of cherry on an artificial medium. Since then, the procedure has been applied in embryo rescue of many other crops, such as *Lilium* (Proseviçius and Strikulyte, 2004), *Gossypium* (Mehetreand Aher, 2004), *Malus* (Dantas *et al.,* 2006), *Prunus* (Kukharchyk and Kastrickaya, 2006), *Elaeis* (Alves *et al.*, 2011), various tree fruits (Fathi and Jahani, 2012) and *Capsicum* (Debbarama *et al.,* 2013).

Application in Protoplast Culture Technology

Protoplasts are described as naked plant cells obtained through the removal of the cellulosic cell wall. The potential use of protoplast technology for the genetic improvement of many agricultural crops is immense. This technology has allowed not only intraspecific hybridization to take place, but also the creation of interspecific and intergeneric hybrids as well as cybridization. Various desirable traits from donor plants have been successfully transferred to hybrids and cybrids using this technology (Gunashree and Venkateswaran (2010); Srinivasan *et al.,* (2009).

Development of Biotic Stress Tolerant Plants through In Vitro Selection

The yields of many commercially important crops remain relatively low due to susceptibility to various fungal, bacterial and viral pathogens. Chemical control of these pathogens is often difficult, costly and labor and resource-intensive (Bezier *et al.,* 2002). In addition, some chemically synthesized fungicides can cause environmental pollution, being non-biodegradable they can build up heavy concentrations in soil reducing its productivity and in the water table posing health hazards to flora and fauna. Hence, studies on development of biotic stress-tolerant plants through existing or novel methodologies have become increasingly important. In vitro selection is an attractive alternative approach for development of stress tolerant lines (Jayashankar *et al.,* 2000; Ganesan and Jayabalan, 2006).

In Vitro Plant Selection for Improving Disease Resistance

In vitro selection through enhanced expression of pathogenesis-related (PR) proteins, antifungal peptides or biosynthesis of phytoalexins is an important tool for desirable plant selection (Ganesan and Jayabalan, 2006; Kumar *et al.,* 2008a). This technology is easy and cost effective compared to the transgenic approach for the improved disease tolerance (Jayashankar *et al.,* 2000). In vitro selection for resistance to a pathogen can be carried out using organogenic or embryogenic calli, shoots, somatic embryos or cell suspensions by exposing them to toxins produced by the pathogen, pathogen culture filtrate or to the pathogen itself (Kumar *et al.,* 2008a).

The possibility of in vitro selection for disease resistance was first reported by Carlson (1973) in tobacco for Pseudomonas syringae. Since then, lines resistant to fungal, bacterial and viral pathogens have been isolated in many species (Fuime and Fuime, 2003; Krause *et al.,* 2003; Gayatri *et al.,*2005; Ganesan and Jayabalan, 2006; Kumar *et al.,* 2008a). In recent years, pathogen culture filtrate and phytotoxins are most commonly used for in vitro selection and regeneration of disease resistant plants in many crops (Kumar *et al.,* 2008a). In vitro selection by adding host-specific phytotoxin such as fusaric acid and pathogen produced nonspecific phytotoxins i.e. deoxynivalenol (DON), crude pathogen culture filtrate or sometimes the pathogen itself to the growth media has been reported to increase the frequency of resistant plants, as compared with those obtained from tissue culture without selection (Gayatri *et al.,* 2005).

Disease resistance has been identified following in vitro selection in a wide range of plant species including cereals, vegetables, fruits and other commercially important plant species (Table 1). In vitro selection of disease resistant lines by using culture filtrate of pathogens have been effectively carried out in herbaceous plants including maize, potato, alfalfa, barley and rice. Some successful reports of in vitro selection for disease resistance in woody species involve peach, lemon, mango and grapes. Hammerschlag (1988) regenerated disease resistant plant of peach by screening embryogenic callus obtained from zygotic embryos against culture filtrate produced by a pathogenic bacterium *Xanthomonas campestris* cv. pruni. The nucellar embryogenic cultures of two polyembryonic cultivars of mango selected against the culture filtrate of *Colletotrichum gloeosporioides* exhibited resistance to the fungus in-vitro (Jayasankar and Litz, 1998). On similar lines, Jayashankar *et al.,* (2000) screened proembryogenic mass of grapes against culture filtrate produced by *Elsinoe ampelina*, the causal agent of anthracnose disease and reported that regenerated plants showed enhanced resistance to the pathogen. Such

studies have also been shown to be useful assays in testing for resistance in wheat (Yang *et al.,* 1998), tomato (Fuime and Fuime, 2003), flax (Krause *et al.,* 2003), turmeric (Gayatri *et al.,* 2005), cotton (Ganesan and Jayabalan, 2006), safflower (Kumar *et al.,* 2008a), sugarcane (Sengar *et al.,* 2009), etc.

In Vitro Selection For disease Resistance by Using Phytotoxin

In vitro selection by using phytotoxin has also been carried out by several workers. Cell suspension cultures of 'Peter Pears', a cultivar of *Gladiolus* × *grandiflorus* (Hort.), susceptible to the fungus *Fusarium oxysporum* f. sp. gladioli (Mass.), have been selected against fusaric acid, one of the toxins produced by this pathogen (Remotti *et al.,* 1997). Similarly, the calli of two genotypes of barley were used for selection of resistance using fusaric acid (Chawla and Wenzel, 1987). Gentile *et al.,* (1993) regenerated 'malsecco' resistant lemon by screening embryogenic cultures of nucellar origin against a partially purified phytotoxin produced by Phomatracheifila. *Fusarium graminearum* tolerant planlets of *Triticum aestivum* L. were successfully screened using deoxynivalenol as a selection agent in vitro (Yang *et al.,* 1998). Toyoda *et al.,* (1989) selected tobacco mosaic virus resistant tobacco in vitro using callus lines infected with tobacco mosaic viruses itself.

Characterization of Disease Resistant Plants during In Vitro Selection

The mechanisms for obtaining disease resistance through in vitro selection remain unclear; however, some authors believed that resistance may be induced in susceptible cells by application of selection pressure. Localized infection of plant tissue is known to result in systemic acquired resistance (SAR) by activation of defense related genes which can be effective against pathogens (Jayashankar *et al.,* 2000). It has been demonstrated that synthetic chemicals such as salicylic acid can also induce SAR in plants (Metraux *et al.,* 1990). It has also been shown that PR proteins that are produced by pathogen are sufficient to induce such SAR in host plants (Strobel *et al.,* 1996). Chitinases and β-1, 3-glucannases are the most important PR proteins expressed by diseased plants. Recent reports have shown that activation of chitinase gene has been widely used for improving disease tolerance in plants (Ganesan and Jayabalan, 2006). Chitinases are the defense enzymes that degrade chitin, a constituent of the fungal cell wall which is not found in plants (Khan and Shih, 2004). Generally, chitinases are induced by several factors like infection by viruses, viroids, fungi and bacteria, application of ethylene, salicylic acid, acetylsalicylic acid and exposure to UV light (Ganesan and Jayabalan, 2006). Jayashankar *et al.,* (2000) speculated that constitutive induction of several detoxifying enzyme(s) during in vitro selection led to systemic resistance in the selected lines.

Recently, Kumar *et al.,* (2008a) reported that the fungal culture filtrate induced oxidative stress during in vitro selection. The oxidative damage is characterized by the activation and deactivation of many ant oxidative defense enzymes such as superoxide dismutase, peroxidase, catalase, and ascorbate peroxidase and glutathione reductase. Ant oxidative enzymes play a key role in the defense mechanism against pathogen culture filtrate or pathogen itself. During pathogen attack, receptor-induced signaling activates membrane or apoplast localized oxidases (NAPDH- oxidase or amine oxidase) that produce superoxide radicals (O2-) that are highly toxic and can help to kill the invading pathogen. These O2-is rapidly dismutated into hydrogen peroxide (H2O2) by superoxide dismutase (SOD). In contrast to O2-, H2O2 can diffuse into cells and activate many of the plant defenses, including programmed cell death (PCD) (Hirt, 2004). Therefore, during plant-pathogen reactions, levels of the ROS detoxifying enzymes like APX and CAT are suppressed, which is crucial for the onset of PCD. Intracellular ROS levels increase not only due to extracellular production of ROS but also by down regulation of ROS scavenging mechanisms. However, the activities of ROS detoxifying enzymes (SOD and POD) was higher in fungal culture filtrate (FCF) tolerant plants of safflower compared to the control plants, which could be considered as a response of FCF induced oxidation damage (Kumaretal, 2008a).

In Vitro Plant Selection for Improving Insect Pest Resistance

Although there have been many notable successes in conventional breeding for improved plant resistance to insects, the breeding process is often slow and laborious, and sufficient levels of resistance have not been achieved for some pests. However, recent progress in plant transformation technologies has made it possible to produce new genetically modified cultivars with improved resistance to insect pests by genetic engineering. In addition, with advances in biotechnology, breeding of horizontal resistance, whereby resistance is based on many genes, along with genetically enhanced sustainable pest resistance with fusion genes, offer new

strategies in improving plant insect resistance (Wan 2006).

In Vitro Plant Selection for Improving Nematode Resistance

Genomic tools are enabling significant progress in the understanding of nematode diseases (Bella fiore and Briggs, 2010). Genome-wide expression pro filing of infected plants has revealed genes that respond to infection and functional tests show they can mediate the interaction with nematodes. Several candidate effectors from nematodes have been identified and functional tests using RNAi have supported their putative roles in pathogenesis. These will increase the possibility to design novel approaches to developing crops resistant to nematode injuries.

InVitro Breeding for Herbicide Tolerance

Depending on the crop and location, weeds can decrease crop yields by 35%–100%. A number of options are available to farmers for minimizing the impact of weeds on crop productivity; one of these is the application of herbicides to the weeds. Indeed, effective weed control is a prerequisite in any crop production system if high yields and good quality are to be achieved, and herbicides have revolutionized weed control in many cropping systems and play an important role in modern agriculture. They provide economical weed control and increase the efficiency of crop production. A number of new herbicides combine high weed killing potency with low- or no-environmental persistence. However, the very effective broad spectrum herbicides available also lack selectivity, thus limiting their use in some cropping operations. On the other hand, the continuous use of the few available selective herbicides is speeding up the development of herbicide resistance in weeds; hence making effective control difficult to achieve in some crops. Biotechnology techniques such as *in vitro* cellculture, mutagenesis and selection in physiologically inhibitory concentrations of herbicides (also referred to as brute force selection) or genetic transformation of already existing crop cultivars with genes that confer resistance to herbicides.

Cell Culture and Selection

Herbicides that interfere with basic metabolic activities are expected to inhibit growth of cultured cells as well as of the whole plant. In such instances, herbicide tolerant mutants can be selected by culturing cells in the presence of a herbicide concentration that is toxic to normal cells, favoring subsequent identification of the herbicide-tolerant target enzyme. Using cell culture techniques, BASF Inc. produced a maize hybrid that is resistant to the sulfonylurea herbicide, sethoxidim. In their analysis, a mutant cell line (named S2) was identified following continuous culture of maize embryo tissues under high sethoxidim selection pressure. Plants regenerated from this somaclonal mutant line were found to contain a form of the enzyme, acetolactate synthase (ALS, target of sulfonylureas/imidazolinones), which was insensitive to the herbicide. This resistance was subsequently transferred to the commercial hybrid (DK404SR) by backcrossing the S2 line with both of its parental lines.

Further investigations showed that the sethoxidim tolerance was inherited as a single partially dominant allele. Similarly, Zambrano *et al.,* (2003) selected a glyphosate-tolerant sugar cane cell line in liquid medium containing 0.8 mM glyphosate and regenerated plants that could tolerate up to five-fold the concentration of glyphosate that killed plants from unselected cells.

Cell culture under lethal concentrations of certain herbicides also results in gene amplification in surviving cells that leads to resistance through the overproduction of enzymes targeted by herbicides.

For example, a petunia cell line with resistance to glyphosate was selected in this manner and plants regenerated from it survived lethal levels of glyphosate (Steinrucken and Amrhein, 1986). This resistance was found to be due to amplification of the gene encoding 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase that caused its overproduction in the cells. Similarly, Caretto *et al.,* (1994) selected carrot cells and subsequently regenerated plants that were resistant to the sulfonylurea herbicide, chlorsulfuron. Resistance in these plants was due to amplification of the *ALS* gene.

In vitro development of phosphinothricin (PPT) resistant rice has also been reported by inducing plantlet regeneration in explants collected from 7-day old seedlings on medium supplemented with sub lethal doses of PPT (Toldi *et al.,* 2000). Other *in vitro* cell selection studies have developed resistance to paraquat in tomato cells (Thomas and Pratt 1982), resistance to glyphosate in carrot and groundnut cells (e.g. Jain *et al.,* 1999) and resistance to a Protoporphyrinogen oxidase (PPO) inhibitor in soybean cells (Warabi *et al.,* 2001) ; however, no viable plant regeneration was reported in these studies.

Table.1 Screening and in vitro selection for disease resistance

Plant species	Selecting agents	Resistant against pathogen	References
***Arachis hypogaea* (groundnut)**	CF	*Cercosporidium personatum*	Venkatachalam and Jayabalan (1996)
***Brassica napus* (rapeseed)**	PT	*Phoma lingum*	Sacristan (1982)
***Carthamus tinctorius* (safflower)**	CF	*Alternaria carthami*	Kumar *et al.*, (2008a)
***Citrus limon* (lemon)**	CF	*Phoma tracheiphila*	Gentile *et al.*, (1992, 1993
***Curcuma longa* (turmeric)**	CF	*Pythium graminicolum*	Gayatri *et al.*, (2005)
***Gladiolus grandiflorus* (gladiolus)**	Fusaric acid	*Fusarium oxysporum*	Remotti *et al.*, (1997
***Glycine max* (soya bean)**	CF	*Septoria glycines*	Song *et al.*, (1994)
***Gossypium hirsutum* (cotton)**	CF	*Fusarium oxysporum*, *Alternaria macrospora*	Ganesan and Jayabalan (2006)
***Hordeum vulgare* (barley)**	Fusaric acid	*Fusarium* sp.	Chawla and Wenzel (1987
***Linum usitatissimum* (flax)**	CF	*Fusarium oxysporum*	Krause *et al.*, (2003)
***Lycopersicon esculentum* (tomato)**	CF	*Pyrenochaeta lycopersici*	Fuime and Fuime (2003)
***Mangifera indica* (mango)**	CF	*Colletotrichum gloeosporioides*	Jayasankar and Litz (1998
***Medicago sativa* (alfalfa)**	CF	*Fusarium oxysporum*	Hartman *et al.*, (1984
***Musa* sp. (banana)**	Fusaric acid	*Fusarium* sp.	Matsumoto *et al.*, (1995
***Nicotiana tabacum* (tobacco)**	Methionine sulfoximine/CFP	*Pseudomonas syrigae* Pseudomonas and Alernaria tobacco mosaic virus (TMV)	Carlson (1973) Thanutong *et al.*, (1983) Toyoda *et al.*, (1989
***Oryza sativa* (rice)**	CF	*Helminthosporium oryzae*	Vidhyasekaran *et al.*, (1990
***Prunus persica* (peach)**	CF	*Xanthomonas campestris*	Hammerschlag (1988)
Saccharum sp. (sugarcane)	CF	*Colletotrichum falcatum*	Sengar *et al.*, (2009)
***Solanum tuberosum* (potato)**	CF	*Phytophthora infestens*	Behnke (1979)
***Triticum aestivum* (wheat)**	DON	Fusarium sp, *Fusarium graminearum*	Maier and Oettler (1992) Yang *et al.*, (1998
***Vitis vinifera* (grapes)**	CF	*Elsinoe ampelina*	Jayashankar *et al.*, (2000)
***Zea mays* (maize)**	PT	*Helminthosporium maydis*	Gengenbach *et al.*, (1977)

CF: culture filtrate, DON: deoxynivalenol, P: pathogen, PT: phytotoxin.

Future Issues

Plant pests and diseases have major effects on agricultural production and the food supply. Although application of fungicides and pesticides has helped control of plant diseases, chemical control is economically costly as well as environmentally undesirable. The development of new strategies based on a plant's own defense mechanisms for biotic stress control is therefore critical for sustaining agricultural production and improving our environment and health. In recent years, considerable progress has been made regarding the development and isolation of biotic stress mainly disease tolerant cell/callus lines using in vitro technique. In vitro selection will save the time required for developing disease resistant and abiotic stress tolerant

lines of important plant species. In vitro selected variants should be finally field-tested to confirm the genetic stability of the selected trait (Rai *et al.,* 2011). The problem in using tissue culture technique is loss of regeneration ability during in vitro selection which can be overcome by the use of explants with high morphogenic potential which may ensure successful regeneration. Epigenetic adaptation is another obstacle for the selection of rare mutants with true tolerance, which can be prevented by the use of short-term or one step selection (Tal, 1994). Despite these problems, many reports are also available on successful regeneration of plants from selected cell/callus lines showing stability in stress tolerance in whole plant. In vitro selection incorporated with molecular and functional genomics can provide a new opportunity to improve stress tolerance in plants relevant to food production and environmental sustainability.

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