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In Vitro Mutagenesis and Selection in Plant Tissue Cultures and their Prospects for Crop Improvement

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ABSTRACT

Mutation induction has become a powerful tool for developing new and novel plant germplasm. Methods such as gamma ray irradiation, ion beams and chemical mutagens have been applied to induce mutations. Since availability of a large number of mutagenized populations for screening and methods of selection are still a hindrance with conventional mutagenesis, *in vitro* mutagenesis of cultured explants, cells and tissue cultures represent a feasible method for induction of genetic variability. Selection at the cellular level has been practised for desirable traits and success has been achieved in several crop plants. This article outlines the different aspects of *in vitro* mutagenesis and selection for varied applications in crop improvement.

Keywords: crop improvement, *in vitro* culture, *in vitro* selection, mutagenesis
Abbreviations: **AEC**, (s-(beta-aminoethyl)-cysteine); **CF**, culture filtrate; **dES**, diethyl sulfate; **DH**, double haploidy; **DON**, deoxynivalenol **EI**, ethylamine; **EMS**, ethyl methane sulfonate; **ENH**, ethyl nitroso urea; **ENU**, ethyl nitroso urethane; **FA**, fusaric acid; **HIB**, heavy ion beam; **HmT**, *Helminthosporium maydis* toxin; **LET**, linear energy transfer; **LIB**, low energy ion beam; **MIC**, maximum inhibitory concentration; **MNH**, methyl nitroso urea; **PEG**, polyethylene glycol; **TILLING**, target-induced local lesions in genomes

CONTENTS

INTRODUCTION

Plant breeding methods over the past several decades have contributed immensely to develop genetically improved crop varieties for increasing food security. These methods continue to enrich the germplasm base of crop plants by evolving genetically superior varieties for cultivation. However, the current population increase demands to embrace new and innovative technologies. From the present 208 million tonnes of food grain production, we may need about 340 million tonnes by the year 2020 to feed the ever-increasing population (Toru and Matoh 2009). Further increase in agricultural productivity equitably in an environmentally sustainable manner, in the face of limiting resources, is a challenging mission.

The use of induced mutations has played a key role in the improvement of superior plant varieties (Ahloowalia 1998; Jain 2005; Maluszynski *et al.* 2004). More than 3000 improved mutant varieties have been released for commercial cultivation in different crop species demonstrating the economic value of the mutation breeding technology (Kharkwal and Shu 2009; Jain and Suprasanna 2011). In addition to the currently practiced methods of genetic improvement, there is a greater need for developing new and innovative research for developing sustainable agriculture systems. The techniques of biotechnology, which include cell culture and molecular biology, have generated great interest in addressing these problems, and in the past decade, integration of both has shown substantial success (Sharma *et al*. 2002). Compared to crossbreeding methods, mutagenesis has the ability to modify only a very few characters in an otherwise promising cultivar without altering significantly the remaining and often unique genetic background. Mutation breeding, therefore, can be considered as a viable option to genetically modify existing commercial clones and, mutagenesis using *in vitro* plant cell and tissue cultures offers as a feasible method in generating novel genetic variability (Larkin and Scowcraft 1983; Brar and Jain 1998). Mutation techniques have also been integrated with other molecular technologies, such as molecular marker techniques and high throughput mutation screening techniques thereby becoming more powerful and effective in crop breeding (Shu 2009).

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Invited Review

CONSIDERATIONS ON USING *IN VITRO* CULTURES

In conventional mutagenesis, limitations exist such as availability of a large mutagenized population for screening and proper selection methods. Irradiation of seeds and vegetative tissues lead to competition among meristematic tissues between lethally and sub-lethally affected cells on one hand and unaffected cells on the other hand, offering the advantage of the latter. Complex nature of apical meristems and propagating materials also pose a problem in vegetatively propagated plants (Micke *et al*. 1990). In this regard, the advantages of *in vitro* mutagenesis include, high mutation frequency, uniform mutagen treatment, application of selective agents to homogenous cell population, use of single cell systems, requirement of less space to handle large population within short time and keeping the plant material disease free. In general, in order to select for variant clones, cells or callus are mutagenized and exposed to specific conditions which allow for the survival of only a small fraction of the population presumptively consisting of spontaneous mutants adapted to these conditions. These conditions include: high concentration of metabolites, toxic drugs, metabolite analogues, absence of essential nutrients or hormones, environmental stress, etc. On the other hand, *in vitro* selection technology combined with spontaneous or active mutagenesis has been effective in altering or isolating genetic variability for characteristics expressed at the isolated-cell level (Maliga 1984; van Harten 1998; Suprasanna *et al*. 2010).

One of the major drawbacks of mutation breeding in higher plants is the formation of chimeras following the mutagenic treatment of multicellular organisms. Cell culture methods of mutant selection are more efficient (Maliga 1984). *In vitro* technique was utilized for isolating new ornamental varieties through retrieval of chimeric tissues derived by induced mutagenesis in chrysanthemum by Datta and Chakrabarty (2009), who proposed that this technique has practical importance not only for chrysanthemum but for other ornamentals also. Also, the combined methods of irradiation and *in vitro* culture yielded a mutation rate eight times higher than the conventional chronic cutting method, also producing non-chimeric mutants in chrysanthemum (Nagatomi and Degi 2009). Lines that are selected *in vitro* are referred as variants which can be studied for the cause of the phenotypic change (mutation or epigenetic change). The term mutant is used only when genetic basis for mutation has been confirmed (Maliga 1984).

MUTAGENIC AGENTS

The most commonly used physical mutagens are ionizing radiation, such as gamma rays (γ-rays), X-rays and fast neutrons. Several types of ionizing radiation, i.e. X- and gamma rays, alpha and beta particles, protons and neutrons, produce the ability called ionization or ion pairs, as they pass through subject matter. Gamma rays have generally a shorter wavelength and hence possess more energy. In general, Cobalt-60 and Cesium-137 are the main sources of gamma rays used in mutation induction. Ultraviolet light has limited penetrating ability; therefore its use is limited to treating spores, pollen grains cells and cultured tissue. The effectiveness of radiation treatments depends heavily on the moisture and oxygen content of the treated material.

Ion beams can give a large amount of energy with high LET (Linear Energy Transfer) to the localized position in tissues. Also ion beams can produce large structural changes in chromosomes and DNA. Compared to ionizing radiations (gamma- and X-rays), it is possible to induce different kinds of mutations in plants with high frequency. By utilizing ion beams, Kirin Agribio has generated many varieties of ornamental plants including carnations, chrysanthemums and petunias (Okamura *et al.* 2001, 2003). In the case of carnations, the parental leaf tissues were irradiated with carbon ions and then the plants were regenerated from them and a great number of flower mutants including unprecedented round-petal carnations were obtained and some of the new varieties have been commercialized as 'Ion Series' varieties (Okamura *et al.* 2001). Reyes-Borja *et al.* (2007) employed carbon-ion beam for *in vitro* irradiation to induce genetic variability for black Sigatoka resistance in banana. Plants resistant to black Sigatoka from 'Williams' and 'Cavendish Enano' population were selected in the field, suggesting that carbon-ion beam could be useful for mutation breeding in banana.

The ion beam irradiation technique has demonstrated good applicability in the induction of novel mutations and plant types (Abe *et al.* 2007). The advantages include low exposure levels, high mutation rates, and a wide variation of mutation. These not only involve energy transfer (as gamma or X-rays), but also mass deposition and charge exchange; hence could result in complex DNA damage and changes that are not found when gamma or X-rays are used (high percentage of double strand breaks and subsequent chromosome aberrations). Ion beams are produced by particle accelerators, i.e. cyclotrons. New rice and wheat mutant varieties have been bred using ion beam technology and released for large scale commercial production in China. Heavy ion beam (HIB) and low energy ion beam (LIB) have been employed for mutation induction in a wide range of crops. Use of HII (accelerated heavy-ion) technology for isolation of induced mutants in many plant species is extensively reported (Abe *et al.* 2000; Tanaka 2009; Jain 2010). In ornamental plants, such as verbena (Suzuki *et al.* 2002), petunia (Miyazaki *et al.* 2002), carnation (Okamura *et al.* 2003) and pepper (Honda *et al.* 2006) new HII-induced cultivars have been developed and made available commercially.

The spectrum of chemical mutagens for mutation induction is abundant and the list of these mutagenic chemicals is ever increasing. Mutagens belonging to the class of alkylating agents are mostly used such as ethyl methane sulfonate (EMS), diethyl sulfate (dES), ethylamine (EI), ethyl nitroso urethane (ENU), ethyl nitroso urea (ENH), and methyl nitroso urea (MNH). In several cases of *in vitro* chemical mutagenesis, explants and calli are treated with MNNG (80 mg l^{-1}), EMS (0.5%), NaN_2 (0.1M) and N_2H_2 (0.05M) (Bourhamont and Dubin 1986). EMS is generally used in a concentration range of 0.2 to 1% whereas the range for nitroso-ethyl urea is 0.1 to 0.3 mM (Deane *et al*. 1995). Several factors including chemical and physical properties, reactivity and solubility of the mutagens, temperature, light and pH of the solution, oxygen availability during the treatment, uptake, application methods and size of the material to be treated besides the post-treatment washing methods of the mutagenized material can modify and affect the outcome of the use of chemical mutagens (Novak 1991). Chemical mutagens are extremely toxic henceforth require more care in their application, compared with physical mutagens. Chemical applications *in vitro* in comparison to physical mutagen are less practical and up to 90% of released *in vitro* mutant varieties are derived from radiation-induced mutations (Micke *et al.* 1990). Nevertheless, there have been examples of increased mutation induction frequency (Muller and Grafe 1978). Soybean and carrot cells treated with EMS and NTG showed 10-fold increased frequency of 5-fluorouracil and cyclohexamide resistant lines (Sung 1976). Widholm (1977) demonstrated a 10-fold increase in the frequency of 5-methantryptophan resistance in EMS treated carrot cells. There were no significant differences between the numbers of variations induced by different mutagens (sodium azide, diethyl sulphate and ethylmethanesulphonate) in *in vitro* grown shoot apices of banana (Bhagwat and Duncan 1998).

IN VITRO CULTURE

In vitro culture techniques are particularly relevant for mutagenesis as totipotent plant cells are cultured, proliferated in large volume and can be induced into regeneration

of complete plants. The different plant material that can be irradiated/mutagenized include rooted stem, cuttings, detached leaves, dormant buds/plants, shoot apices (apical buds), axillary buds, tubers, etc. One of the prime considerations of using *in vitro* cultures for mutagenesis is based upon the fact that large populations of cells can be treated and screened before being regenerated into complete plants (van Harten 1998). Callus and cell suspension cultures that show good regeneration potential offer as an attractive target source. Among the different *in vitro* methods, somatic embryogenesis is the most useful tool for mutagenesis as somatic embryos usually originate from single cells. Furthermore, a number of subcultures can be performed in a short time for chimera separation and to increase the mutagenized population for selection. Consequently, non-chimeric mutants can be isolated from the irradiated explants through callus proliferation. The possibilities are much higher for obtaining such desired mutants if cultures can be induced into secondary embryogenesis or repetitive embryogenesis. *In vitro s*ubcultures are usually carried through M_1 (irradiated explants) for three and in some cases 4-6 cycles. The major factors that can influence during the regeneration process are mutagen treatment *per se*, the gene affected or trait selected and expressed during the selection and the *in vitro* culture passage. Optimized mutation – selection conditions combined with an early regeneration of selected variants can reduce the time required for regeneration.

Haploid callus cultures derived from microspores / ovules are also the choicest targets for mutagenesis. Haploid cell and protoplast cultures have advantages in studies on mutant selection *in vitro*, since mutations particularly recessive in nature can easily be detected in the subsequent generations and, the ability to fix mutations via doubled haploidy (DH) is a key factor, especially as induced mutations are predominantly recessive and cannot normally be detected until the M_2 generation at the earliest (Szarejko and Forster 2007). Mutagen treatment can be given at different stages: at the parent cultivar stage so that M_1 plants are used for culturing microspores or anthers for subsequent selection and doubled haploid mutant or M1 plants are developed from which haploid explants are cultured for obtention of doubled haploid mutant lines. Using the microspore culture combined with mutagenesis, selection for tolerance to herbicides (chlorosulphuron, imidazolinone), resistance to blackleg, high oleic acid and low linoleic acid and low level of saturated fatty acids has been successfully accomplished through haploid embryos followed by haploid plants and doubled haploids (Szarejko *et al.* 1991; Maluszynski *et al.* 1995).

Anther culture followed by mutagenesis can enable fixing of recessive mutations and stable mutants after diploidization. The advantages of a microspore-based selection system include the use of large populations of single haploid cells, a low level of somaclonal variation, the opportunity for efficient and uniform mutagen application, immediate expression of recessive traits, and homozygosity of selected DH mutants (Swanson *et al.* 1989). Medrano *et al.* (1986) isolated numerous chlorophyll mutants by EMS treatment of *Nicotiana tabacum* anthers. Anther cultures of *japonica* rice treated with EMS or EI resulted in many morphological mutants (Hu 1983). Haploid somatic cells have also been treated with EMS and EI, and morphological mutants were isolated in *Nicotiana sylvestris* and *Brassica napus* (Malepszy *et al.* 1977: Hoffmann *et al.* 1982). Anther culture for the production of haploids is well established in *Brassica* species and the technology has been utilized for various applications in crop improvement (Babbar *et al.* 2004). Doubled haploid line having mutations for altered fatty acid composition (increased oleic acid level and reduction in linolenic acid) have been isolated in *Brassica* (Wang and Swanson 1991). EMS treatment given to rice anthers 10 days after culture yielded high frequency (20%) of stable mutants for semi-dwarf, grain-shape and glabrous traits (Lee and Lee 2003).

The problems of recovering mutations in vegetatively propagated plants have been attributed to the phenomenon 'intrasomatic selection', because of the more complex nature of apical meristems and propagating materials. Intrasomatic selection (Kaplan 1951) or diplontic selection (Gaul 1961) is the competition that occurs between lethally and sub-lethally affected cells, when seeds and vegetative tissues are irradiated. To minimize such effects in vegetatively propagated plants, chronic irradiation, neutron irradiation, chemical mutagenesis and lastly, mutagenesis of isolated single cells and growing them into whole plants can be useful (Nayyar 1969). Lower regeneration response at higher doses as observed in general in radiation mutagenesis studies could possibly be attributed to toxic effect of gamma radiation on cells / tissues and less competitiveness of these cells and their progenies. Such a response has been noted in several *in vitro* mutation induction experiments (van Harten 1998). Studies using sugarcane embryogenic callus cultures, higher-dose gamma-irradiated embryogenic cultures displayed poor or no regeneration potential. In this regard, it was considered to use culture treatments or media manipulations to elicit regeneration response. The high-dose irradiated embryogenic cultures were subjected to partial desiccation for 4-6 h to stimulate and enhance somatic embryo differentiation and plant regeneration response (Suprasanna *et al*. 2008b). Intrasomatic competition discriminating mutagen affected cells and potentially causing a loss of their cell progenies may also be controlled by modifying *in vitro* conditions (medium composition or some other factors) resulting in a better competitiveness of mutant cells (van Harten 1998). The partial desiccation method (Suprasanna *et al*. 2008b) could be useful as a simple method in stimulating regeneration response in case of mutagenized cultures.

IN VITRO SELECTION

The selection and identification of desirable mutants are an integral part to any mutation-breeding programme. As compared to methodologies involving treatment of *in vivo* material, *in vitro* cultured explants provide a wider choice of controlled selection following mutagenic treatment. Screening performed *in vitro* allows handling of large populations, avoiding the problem of working with a low number of individuals as in the case of *in vivo* plant material. In this regard, mutagenized cell suspension cultures and protoplast cultures can be of great advantage owing to their more genetic uniformity than calli, embryos, and other explants. The achievement of *in vitro* selection technique to obtain tolerant plants requires the availability of: (i) high variation of cells, (ii) easy application of *in vitro* selection method, (iii) regeneration method of tolerant cells (Widoretno *et al.* 2003), and (iv) the desired character to be inherited (Yusnita 2005).

In any selection scheme, it is advantageous that the trait of interest be selectable at the cellular level and express in the regenerated plants. However, not all the traits are selected at the cellular level, for example, yield, seed color or plant height, which are mostly under polygenic control (Ahloowalia 1998). On the other hand, some traits of agronomic importance and some with a fundamental interest can be selected using selection agents in plant cell cultures. Disease resistance, stress tolerance particularly for salt and drought, enriched nutritional quality and herbicide tolerance are some of the traits selected *in vitro.* Mutants have been induced and recovered in several plant species (Predieri 2001).

During *in vitro* selection, two types of selections viz. single step selection and multi-step selection are practiced. Generally, an inhibitor or an antimetabolite is added into the culture medium at a level that will either kill or inhibit the growth of the mutagenized cells. In the single step selection, the inhibitor is added into the culture medium, at least 2-3 times the level of maximum inhibitory concentration (MIC) and cultures are maintained for several subculture regimes.

Table 1 Examples of *in vitro* selection for abiotic stress tolerance in crop plants.

Plant species	Selection agent and level used	Tolerance to selectable trait	Reference
Saccharum sp.	Mannitol (0.62, 0.84 and 1.08 MPa)	Drought	Errabii *et al*. 2006
Oryza sativa	PEG (control and 100 gL^{-1})	Drought	Adkins *et al*. 1995
Capsicum annuum L.	PEG (0, 5, 10, 15, 20, 25 or 30% PEG and gradually decreased to 0% by continuous sub-culturing)	Drought	Santos-Diaz and Ochoa-Alejo 1994
Durum wheat	PEG 10000 (molecular weight)	Drought	Hsissou and Bouharmont 1994
Tagetes minuta	Mannitol (6-80 mM)	Drought	Mohamed *et al.* 2000
Brassica juncea	NaCl and mannitol (adapted to NaCl (171 mM) and mannitol (329 mM))	Salt and drought	Gangopadhyay *et al*. 1997
Tobacco	NaCl and PEG (*In vitro* selection at 0, 50, 100, 150, 200 mM of NaCl and KCl and 0, 5, 10, 15, 20, 25% of PEG)	Salt and drought stress	Sumaryati *et al*. 1992
Sugar beet	Multiple salt treatment (7.6 g/l of medium (1.6 $NaHCO_3$, 1.2 NaCl, 1.2 $CaCl_2$, 2.0 $MgSO_4$, and 1.6 $CaSO_4$); whole plant culture 60 ml/30 day of 10 mg/ml of the multiple salts ($NaHCO_3$ 2.1, NaCl 1.6, $CaCl_2$ 1.6, $MgSO_4$ 2.7, $CaSO_4$ 2.0 g/l)	Multiple salt stress	Freytag *et al*. 1990
Saccharum sp.	NaCl (42.8, 85.6, 128.3, 171.1, 213.9, 256.7, 299.5 or 342.2 mM)	Salt stress	Patade *et al*. 2008
Oryza sativa L.	NaCl (1 and 2% for *in vitro*; 0.5% for natural conditions)	Salt stress	Vajrabhaya *et al*. 1989
Brassica juncea	NaCl (*in vitro* regeneration at 0.25, 0.5, 0.75, and 1.0% w/v of NaCl and greenhouse evaluation at 0, 30, 60, 90 meq/l of NaCl	Salt stress	Jain *et al*. 1990
Brassica juncea	NaCl (*in vitro* proliferation 0, 1.0, 1.25, 1.50, 1.60, 1.80, 2.0% NaCl)	Salt stress	Kirti *et al*. 1991
Alfalfa	NaCl (regenerated on medium containing 1% NaCl)	Salt stress	Winicov 1991
Vigna radiata	Mannitol (0, 180, 360, 449, 540, 629, 720 $molm^{-3}$ of mannitol)	Drought	Gulati and Jaiwal 1993
Oryza sativa	NaCl (electrical conductivity (EC) levels of NaCl (4.0, 6.0, 8.0 and 10.0 d/Sm)	Salt stress	Saleem *et al*. 2005
Ipomoea batatas L.	NaCl (culture media supplemented with 0, 86, 171, 257 and 342 mM of NaCl)	Salt stress	He *et al*. 2009
Zoysia matrella L.	NaCl (0.3 M)	Salt stress	Chen *et al.* 2011
Oryza sativa	NaCl (0, 0.5, 1.0, 1.5, 2.0% of NaCl)	Salt stress	Shankhdhar *et al.* 2000
Oryza sativa	NaCl (EC at 6 and 12 dS/m by NaCl)	Salt stress	Lee *et al*. 2003
Brassica oleracea	NaCl (0, 85, 170, 255 and 342 mM NaCl)	Salt stress	Elavumoottil *et al*. 2003
Citrus limon	NaCl (0 and 170 mM NaCl)	Salt stress	Piqueras *et al*. 1996
Chrysanthemum morifolium	NaCl (direct and indirect stress at 0, 50, 75 and 100 mM of NaCl)	Salt stress	Hossain *et al*. 2007
Citrus aurantium	NaCl (0, 100, 200 and 300 mM NaCl)	Salt stress	Koc *et al*. 2009
Brassica napus	NaCl ((0, 0.1, 0.2, 0.3, 0.4,0.5, 0.6, 0.7% NaCl)	Salt stress	Rahman *et al*. 1995
Glycine max	NaCl (0, 25, 50, 75, 100, 125, 150 mM NaCl)	Salt stress	Liu and van Staden 2000
Strawberry	NaCl (200 mM NaCl)	Salt stress	Dziadczyk *et al*. 2003
Diplachne fusca	NaCl (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0% NaCl)	Salt stress	Nanakorn *et al*. 2003
Dendrocalamus strictus	NaCl (tolerance screened at 0, 50, 100, 150, 200, and 250 mM NaCl and finally selected at 100 mM NaCl)	Salt stress	Singh *et al*. 2003
Cynodon transvaalensis × *C. dactylon*	NaCl (0.15 and 0.25 M NaCl)	Salt stress	Lu *et al*. 2007
Helianthus annus	NaCl (0 and 175 mM NaCl)	Salt stress	Davenport *et al.* 2003
Vigna radiata	NaCl (0, 50, 100 and 150 mM NaCl)	Salt stress	Hassan *et al*. 2008
Triticum aestivum	NaCl (direct regeneration 0, 3, 6, 9 or 12 g/L NaCl and step-wise increase in NaCl up to 9 g/l)	Salt stress	Barakat and Abdel-Latif 1996
Triticum aestivum	NaCl (2.5, 5, 10 or 15 g/l NaCl)	Salt stress	Zair *et al*. 2003
Solanum tuberosum	NaCl (direct selection 60, 90, 120, 150, 300 or 450 mM)	Salt stress	Ochatt *et al.* 1999
Solanum tuberosum	NaCl (50, 100, 150 or 200 mM NaCl)	Salt stress	Queirós *et al*. 2007
Saccharum sp.	NaCl (0 or 68 mM NaCl)	Salt stress	Gandonou *et al*. 2006
Nicotiana tabacum	NaCl 175 mM	Salt stress	Rout *et al*. 2008
Morus sp.	NaCl (0, 0.25, 0.50, 0.75 and 1.00% NaCl)	Salt stress	Vijayan *et al*. 2003
Medicago sativa	NaCl (screened on 0-350 mol m^{-3} NaCl finally 250 mmol m^{-3} used further analysis)	Salt stress	Safarnejad *et al*. 1996
Lycopersicon esculentum	NaCl (raised from 0 to 15, 30 and finally 50 mM NaCl)	Salt stress	Kripkyy *et al*. 2001
Brassica oleracea var. *botrytis*	NaCl and hydroxyproline (*in vitro* 350 mM and *in vivo* 550 mM NaCl; *in vitro* 3 mM and *in vivo* 10 mM hydroxyproline)	Frost and salt	Fuller *et al*. 2006
Winter barley	Hydroxyproline (10-20 mM)	Frost	Tantau *et al.* 2004
Cymbopogon martinii (Roxb.)	NaCl (300 mM)	Salt stress	Patnaik and Debata 1997
Oryza sativa	Al (0, 250, 500, 7500, 1000, 1250, 1500, 2000 μM of Al)	Aluminium	Jan *et al.* 1997
Oryza sativa	Al (0, 30 and 60 ppm of Al in the form of $Al_2(SO_4)_3 \cdot 18H_2O$)	Aluminium Aluminium	Roy and Mandal 2005 Biswas *et al*. 2002

Examples include selection for herbicide tolerance (Chaleff and Parsons 1984), amino acid enrichment through lysine + threonine resistance (Hibberd and Green 1982), salt tolerance (Bressan *et al.* 1985) and disease resistance (Gengenbach and Green 1975). In a multi-step selection method, a sub-lethal concentration (less than MIC) is added into the medium for *in vitro* cultures to grow and in the subsequent subcultures, a gradual increase in inhibitor level is maintained. With this method, it has been suggested that mutant trait selected will often be more stable and more expressive, since the variant cells are in constant exposure to the increasing levels of the inhibitor (Miller and Hughes 1980; Miao *et al.* 1983; McCoy 1987; Patade *et al.* 2006).

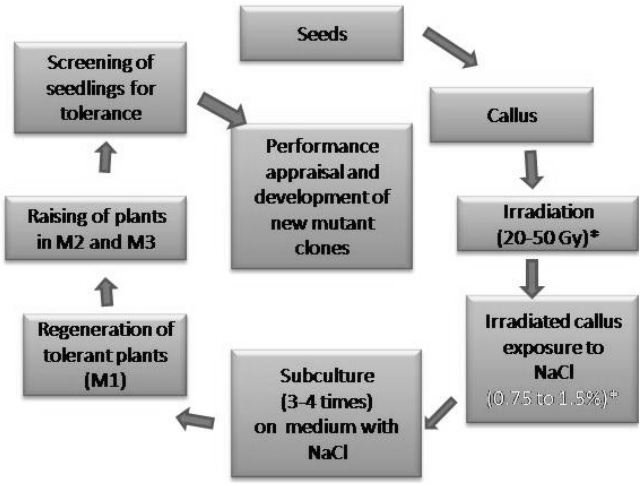

Fig. 1 A generalized scheme for developing salt tolerance in plants using *in vitro* mutagenesis followed by *in vitro* selection [*depending on plant system used].

SELECTION FOR ABIOTIC STRESS TOLERANCE

Development of abiotic stress tolerant plants specially for salt and drought conditions using *in vitro* selection has been reported in a wide range of plant species including cereals, vegetables, fruits and other commercially important plant species (Rai *et al.* 2010). Unlike the conditions in the field or the nursery, a better control of culture environment can be achieved through *in vitro* screening techniques. Salt and drought tolerance has been reported in many plants and in most cases, selection is applied to callus or cell suspension or protoplast cultures by the inclusion of growth inhibitory levels of selection agent (sodium chloride, polyethylene glycol (PEG), sorbitol, mannitol) in culture medium (**Table 1**). A general scheme of *in vitro* selection for salt tolerance is presented in **Fig. 1**. Selection for acid soil and Aluminum tolerance can be made with aluminum chloride as the selection agent on the low acid media as much as pH 4 and the method can be employed in isolating Al-tolerant plants. It is also possible to select cell lines resistant to proline analog to develop mutants with increased free proline and tolerance to stresses such as salt, drought or cold (Widholm 1976).

Both one-step and step-wise selection methods can be applied (Bressan *et al.* 1985; Nabors 1990). In a single step selection, the callus or explant material is exposed once or few times to the inhibitory level of sodium chloride and then resultant surviving tissues are isolated and plants regenerated. Using such a criterion, salt tolerant plantlets have been obtained in flax (Rowland *et al.* 1989), sugarbeet (Freytag *et al.* 1990), *Brassica juncea* (Kirti *et al.* 1991) and sorghum (Waskom *et al.* 1990). The second method is the long-term step-wise selection in which cultures are allowed to grow over several subculture cycles in the presence of high salt concentrations. Bressan *et al.* (1985) obtained salt adopted tobacco cells, which were grown for at least 25 generations in 25 gl^{-1} sodium chloride. Ochat and Power (1989) applied the long-term selection method in colt cherry cell lines that survived six transfers on the same salt or mannitol-containing medium and subjected to three cycles of direct recurrent selection, each consisting of 2-3 week subcultures on salt medium. NaCl-resistant cell lines were also developed from *Nicotiana tabacum* L. cell suspension culture treated by the mutagen EMS and then grown in a medium containing 0.03 M NaCl and then on medium as high as 0.09 M NaCl (Nabors *et al.* 1975). A third approach is the indirect way of selecting for a resistance to proline analogue or ABA insensitivity. Cultured cells of carrot (Ricardi *et al.* 1983), *Brassica napus* (Chandler and Thorpe 1986) and *Vigna radiata* (Kumar and Sharma 1989) exposed to proline analogs, exhibited tolerance to salt stress. Stable NaCl-tolerant chrysanthemum variants were developed through whole plant or callus selection after *in vitro* mutagenesis using ethylmethane sulfonate (EMS) as the chemical mutagen (Hossain *et al.* 2006). Embryogenic suspension cultures of sweetpotato cv. 'Lizixiang' were exposed to 80 Gy gamma-rays followed by *in vitro* selection with NaCl (He *et al.* 2009). A total of 276 plants regenerated from the irradiated 2,783 cell aggregates by a two-step *in vitro* selection with 86, 171, 257 and 342 mM NaCl, of them 18 plant lines showed significantly higher *in vitro* salt tolerance than control plants. Selection *in vitro* has been practiced and tolerant lines have been obtained in several crop plants including brassica, bamboo, sunflower, strawberry, soybean, flax, rice, tomato, potato, sweet potato, sugarcane, wheat and rice (Rai *et al.* 2010).

SELECTION FOR BIOTIC STRESS TOLERANCE

Plant diseases are caused by a variety of different pathogens. Selection systems to isolate tolerant lines have been designed using selection for resistance to culture filtrates, chemicals and toxins (**Table 2**). In a first report by Carlson (1973), this possibility of *in vitro* selection for disease resistance was explored in tobacco for selection against *Pseudomonas syringae* that causes wildfire disease. Several *Helminthosporium* toxins have successfully been used in crop plants. In maize, Genegenbach *et al.* (1977) applied *Helminthosporium maydis* T (HmT) toxin in the selection medium to select embryogenic callus and regenerated resistant plants that exhibited disease resistance. In sugarcane, Larkin and Scowcroft (1981) exposed callus cultures of cultivar Q-101 to *Helminthosporium sacchari* toxin and recovered 480 regenerated plants and several of these were resistant. Chawla and Wenzel (1987) used 3000 callus cultures of barley and 2000 callus cultures of wheat for selection against *Helminthosporium sativum* and in 6-17% of calli and regenerants, resistance was evident. The use of fusaric acid (FA) has also been useful in the selection of *Fusarium*-resistant plants (Remotte and Loffler 1997). Culture filtrates (CF) represent an easy and simple method of selection by incorporation into the culture media at appropriate concentrations. In several selection experiments, culture filtrates, both purified and partially purified, have been successfully used. Lines resistant to fungal, bacterial and viral pathogens have been isolated in many plant species (**Table 2**) by using pathogen culture filtrate and phytotoxins for *in vitro* selection and regeneration of disease resistant plants in many crops (Kumar *et al.* 2008a; Suprasanna *et al.* 2008a; Rai *et al.* 2010).

SELECTION FOR NUTRITIONAL QUALITY

The nutritional quality and stress tolerance of crop plants can be greatly improved if suitable mutants affecting the metabolism or catabolism of essential amino acids could be identified, isolated and developed into new varieties. Induced mutations play an important role enhancing nutritional quality in crop plants. Several mutant genes have been successfully introduced into commercial crop varieties that significantly enhance the nutritional value of crops (Jain and Suprasanna 2010). *In vitro* techniques offer advantages of biochemical selection pressure in the recovery of specific metabolic mutants (Green and Phillips 1974; Suprasanna and Rao 1997). Selected plant cells resistant to amino acid analogs or certain amino acid combinations often have elevated levels of corresponding natural free amino acids. Different types of amino acid analogs have been adopted for selection in cultured plant cells and recovery of resistant cell lines or plants. Cell lines were developed for resistance to *p*-fluorophenylalanine, a phenylalanine analog, which overproduce phenolics due to the presence of increased levels of phenylalanine ammonia lyase (Berlin and Widhom 1977). Kim *et al*. (2004) isolated AEC resistant gamma-ray irradiated rice mutants through *in vitro* mutagenesis, and the high amino acid-accumulating mutant

Table 2 Examples of *in vitro* selection for disease resistance in crop plants.

Crops	Selective agent	Resistance	Reference
Annona comosus	Filtrate, FA	*Fusarium subglutinas*	Borras *et al.* 2001
Arachis hypogaea	CF	*Cercosporidium personatum*	Venkatachalam and Jayabalan 1996
Brassica napus	CF	*Phoma lingam*	Sacristan 1982
	Partially purified CF	*Alternaria brassicicola*	MacDonald and Ingram 1985, 1986
Carica papaya	Sporangial suspension of *Phytophthora palmivora*	*Phytophthora* wilt	Sharma and Skidmore 1988
Carthamus tinctorius	CF	*Alternaria carthami*	Kumar *et al*. 2008a
Citrus limon	CF	*Phoma tracheiphila*	Gentile *et al*. 1992
Curcuma	CF	*Pythium graminicolum*	Gayathri *et al*. 2005
Curcuma longa	CF	*Pythium graminicolum*	Gayatri *et al*. 2005
Fragaria vesca	Partially purified toxins	*Phytopthora cactorum*, *Rhizoctonia fragariae*, *Botrytis cineria*	Battistini and Rosasti 1991; Orlando *et al.* 1997; Remoti 1998
Gladiolus grandiflorus	FA	*Fusarium oxysporum*	Remotti *et al*. 1997
Glycine max	CF	*Septoria glycines*	Song *et al*. 1994
Gossypium hirsutum	CF	*Fusarium oxysporum*, *Alternaria macrospora*	Ganesan and Jayabalan 2006
Hordeum vulgare	FA	*Fusarium* spp.	Chawla and Wenzel 1987
Linum usitatissimum	CF	*Fusarium oxysporum*	Krause *et al.* 2003
Lycopersicon esculentum	CF	*Pyrenochaeta lycopersici*	Fujime and Fujime 2003
Malus domestica	CF	*Phytopthora cactorum*	Utkhede 1986
	Co-cultivation	*Venturia enequalis*	Raman and Goodwin 2001
Mangifera indica	toxin	*Colletotrichum gloeosporioides*	Jaysankar *et al*. 1999
Medicago sativa	CF	*Fusarium oxysporum* f.sp. *medicaginis*	Hartman *et al*. 1984; McCoy 1988
Musa spp.	CF	*Fusarium oxysporum*	Matsumoto *et al.* 1999
Oryza sativa	CF	*Helminthosporium oryzae*	Vidhyasekaran *et al*. 1990
Peach	Fractionated CF	*Xanthomonas campestris* pv. *pruni*	Hammerschlag 1988
Psidium guajava	Cell-free filtrate	*Penicillium vermosonii wilt*	Vos *et al*. 1998
	CF of *F. oxysporum* sp. *solani*	*Fusarium oxysporum wilt*	Bajpai *et al*. 2007
Solanum tuberosum	CF	*Phytophthora infestans*	Behnke 1980
Tobacco	Methionine sulfoximine	*Pseudomonas syringae*	Carlson 1973
Triticum aestivum	DON	*Fusarium* sp.; *Fusarium graminearum*	Maier and Oettler 1992; Yang *et al*. 1998
Saccharum officinarum	Phyotoxin	*Colletotrichum falcatum*	Mohanraj *et al*. 2003
	Toxin	*Helminthosporium sacchari*	Heinz *et al*. 1977; Larkin and Scowcroft 1983
	CF	*Colletotrichum falcatum*	Sengar *et al*. 2009
Vitis vinifera	Dual culture	*Plasmopora viticola*	Barlass *et al.* 1986
	Filtrate	*Bortrytis cinerea*	Reustle and Matt 2000
	CF	*Elsinoe ampelina*	Jayasankar *et al*. 2000
Wheat	Syringomycin	*Pseudomonas syringae* pv. *syringae*	Pauly *et al*. 1987
Zea mays	HmT toxin	*Helminthosporium maydis*	Gengenbach *et al*. 1977

Abbreviations defined on page 1.

lines could be useful in molecular and biochemical studies into the regulation of the improved nutritional quality and abiotic stress tolerance. Ethionine-resistant plants of the forage legume *Astragalus adsurgens* Pall were isolated following mutagenesis with *N*-methyl-*N*-nitrosoguanidine and selection with 0.6 mM ethionine (Luo *et al.* 2005). Cell lines showing 7–8 times more resistance to ethionine, than that of control were obtained and plants regenerated. The results suggested that resistant colony line that could regenerate plants with over-accumulation of methionine might provide an alternative approach to improve the nutritional quality of this forage.

FUTURE PERSPECTIVES

The use of *in vitro* culture techniques in mutation breeding can be integrated into plant improvement programs to derive advantages such as generation of screening population, selection method, chimera separation and increased efficiency of induction genetic variation. Tissue culture, *in vitro* propagation and double haploidy can be employed to increase the efficiency of preparing mutant populations. In addition to their use in generating novel varieties for crop improvement, the use of induced mutations has also become a most valuable resource in understanding genetic, physiological and biochemical basis of trait improvement. There have been substantial technological developments in the induction, screening, and utilization of mutated genes. These include, DNA markers linked to mutated genes for marker-assisted selection and tracing of the gene, and target-induced local lesions in genomes (TILLING), as well as different variant versions for high throughput screening of mutated alleles. With the expanse of genome sequence resource and techniques for modifying specific genes, the area of mutagenesis is passing through a phase of resurgence. Sustained research and awareness about the potentials of mutagenesis will let researchers realize the increasing potential of genetic variation in crop improvement.

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