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REVIEW

IN VITRO CULTURES, SELECTION OF BIOCHEMICAL MUTANT AND PLANT DIVERSITY IMPROVEMENT

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ABSTRACT

Invitro culture and selection of biochemical mutants in plant have brought the development of plant biotechnology become more important. The successful of the *invitro* biochemical mutation methods will be reviewed with several explanations of selection, clones diversity, the use of irradiation and mutagenesis screening methods. The molecular basis concerning DNA mutation will be described out.

INTRODUCTION

Throughout the ages, man has made diverse and increasingly elaborate in using plants. In year of 2000s over 5 billion peoples all over the word will depend on plant. Since plant existing in the world, it has contributed as numerous uses of course, as chemicals factory of natural products for food supply either directly of after animal conversion. Recently dramatic increase of using plant has been reported in pharmaceuticals, cosmetics, perfumes, flower, food supplement and other industrial products. Thus, cereals from the mainstay of human nutrition, alkaloid from poppy and periwinkle are still widely used in pharmaceutical, rose and jasmine flower remain essential ingredients for perfumes, while forestry-based industries are the major source of income for many countries. Without wishing to dwell the importance of plan materials in world trade, it is still worth noting that, after crude oil, such raw materials as coffee, cereals, soya and cocoa are the prime

elements in international monetary exchange, rather than manufactured products.

The efficiency production of plant-derived products depend on many factors, some scientific aspects of the selection for new plant varieties plays a major role and is generally recognized. Variety innovation using traditional techniques has been extremely successful, but rapid development of recent advanced in plant biotechnology has permitted the development of a whole set of new tools.

Traditional methods rely primarily on the combination of interesting characteristics through sexual reproduction. Achieving such sexual reproduction can be regarded as the *technology lack of conventional selection*. This lack of suffers from various limitations: *lack of inter-specific compatibility*, possible instability of the new gene combination during plant propagation as well as a frequently long duration of each sexual reproduction cycle. Biotechnology describe three basic objective issue of concern for practical interest: (1) the increase of genetic sexual reproduction (2) the propagation of genotype which normally are unstable in sexual reproduction (3) the decrease in the number of duration of breeding cycles needed for a selection program.

TECHNIQUES IN PLANT BIOTECHNOLOGY

As mention earlier, the main objectives of plant biotechnology are to eliminate or minimize two key limitations of sexual reproduction (compatibility and stability). There are two types of techniques, those aimed at the modification of genotype, and stabilizing or multiplying a pre-existing genotype. They may be exploited either in the form of whole plant or, remaining at the cellular level.

Firstly is to modify genotype such as genetic engineering, somatic hybridization, and mutagenesis or somaclonal variation. Secondly is to fix or to mutiply a genotype such as haploidization, somatic embryogenesis, and micropropagation

These techniques are to be fruitfully exploited if the techniques of regeneration of plant from protoplast, cells, or tissues must established. There would be no real practical impact of introducing a foreign gene into a plant cells if we were unable to create a stable plant regeneration from this cell. Each individual plant species has varieties of cells regeneration depend on capability adaptation of the technique. There is some mastered model plant protoplasm regeneration such as *solanaceae* has long been use to select some interesting mutant[1-4]. Different situation for legumes and cereals, it will take many years of efforts to achieve plant regeneration from protoplast tissues. Recently, various studies comparing tissues and cells, which can or

cannot be regenerated, have been initiated. The ability to recorver intact plants from cultured cells was originally viewed as an efficient method of producing large numbers of clones, an expectation that has been released for certain plants. Many commercial laboratories use tissue culture of shoot tips to propagate a wide range of ornamental plants including orchids, fern, lilies, *ect*. and some crop plants, such as oil palm. As predicted, when conventional propagation is limited tissue culture propagation has been shown to be economically competitive and has resulted to a large extent in clonal fidelity[5,6]. How ever, it was recognized in our experiences in doing tissue cells cultures that, if cultures were established from explant that did not contain a preorganized meristem, or if cultures were maintained as callus prior to plant regeneration, the regenerated plants were quite variable[4-8].

Skirvin and Janick[9] systematically compared plants regenerated from callus of five cultivars of *Pelorgonium spp*. Plants obtained from geranium stem cutting *invitro* were uniform, whereas plants from *invivo* root and petiole cuttings and plants regenerated from callus were quite variable. This suggest that some variability is correlated with donor explant and pre-existing in the tissue used to establish cell cultures. On the other hand, evidence concentration of the culture medium. Most likely variability is the result of both pre-existing genetic instability and genetic alterations induced during the process of cell culture and subsequent plant regeneration. Clones of generated sugarcane with resistance to Fiji disease virus and downy mildew has been established. These selected resistant clones did not show reduced sucrose yield. Similarly variants of sugarcane have been isolated with resistance to eyespot disease[3,9]. Most sugarcane variants have attributed to changes in chromosome number.

MUTAGENESIS AND INVITRO SELECTION

Genetic variation, which is the basis of plant breeding, will be increased greatly through induced mutation[2,7,10,11]. Use of radiation or chemical mutagen has induced heritable variation to supplement the existing germplasm available for selection, hybridisation and clonal propagation. As explain above that plant tissue culture has made available variation, designed as somaclonal, released under particular in vitro condition[5,6,12]. Part of this variation has proved to be heritable and therefore useful for breeding purpose. Mutation breeding, combined with tissue culture, has made a significant contribution to plant breeding methodology by introducing new techniques for the propagation and induction of genetic variation, by improving the selection technology and by accelerating the breeding time [11,13,14].

The same array of physical and chemical used routinely to induce mutations in seed and vegetative plant parts can be used to induce mutation invitro[5,11]. Application of mutagens during invitro culture requires certain modification, depending on the explant and mutagen used. The following parameters should be considered for physical mutagens (ionizing radiation, UV)[4,14] :

(1) High penetration in multicellular system, excluding UV
(2) The radiation doses and the dose can be measures easily (a high degree of reproducibility).
(3) Radiation can produce chemical changes in the culture media, some radiolysis products have toxic or morphogenetic effect. The transfer to fresh medium after irradiation is important.
(4) Application of fractionated radiation doses can improve the recovery from radiation damage.
(5) The physical parameters of cultivation (temperature, oxygen and media composition) can modify the recovery of irradiated explants and DNA damage and repair in cultured cells.
(6) Chromosome aberations are efficiently induced by ionizing and UV radiation's.

The advanced of using invitro mutation is that large population of individuals (shoot tips, zygotic and or somatic embryos, tissue explant, callus tissue, cells, microspores and protoplast) can be treated before regenerated into intact organism. When reliable protocol for invitro culture and plant regeneration is established, the mutagenesis is performed in several steps[14].

(i) Establishment of dose response curve for the tissue culture involved according to formula :

$$\frac{Xn - Xo}{Xc - Xo} \times 100$$

Where : Xn is average value of dose n; Xc is average value of control; Xo is initial value of inoculum

The fresh or dry weight, cell packed volume, number of cells and planting efficiency may be used to determined growth in response to mutagenic treatment. The selected dose usually reduces growth invitro by about 50-90%[2,7,31,14]. A criterion similar to that used for invitro growth should be applied for the survival rate (viability) and maintenance of the regeneration ability in tissue cultures.

(ii) Following mutagenic treatment, the tissue cultures may subjected to invitro screening and selective selection[4]. Of course, if desired mutant trait cannot be screened selected invitro, and then the plants must be regenerated and evaluated in the greenhouse or in the field.

Selection pressure should not be applied immediately after irradiation/mutagenic treatment. Several cell generations and appropriate recovery period are necessary before the selection experiment is performed. Selection for resistance to abiotic factors in culture medium (salinity, herbicide and amino acid analogues) is the most straightforward method of selecting mutants. Concentrations which are completely (almost killed all the cells) inhibitory to tissue culture growth should be determined. Single step selection is performed with a mutagenized population on media with a two three time's higher factor than the inhibitory concentration[2-4]. The multiple step selection, based on gradually increasing the inhibitory level, is often more successful in obtaining vigorously growing cultures at a high stress concentration. We have established the selection method of several biochemical mutants derived from single cells culture of protoplast[2-4,7].

Frankard[15] has selected and characterised biochemical and genetically high threnine producer mutant from *Nicotiana sylvestris*. Mutagenesis and the subsequent selection of mesophyll diploid protoplast of *Nicotiana sylvestris* on growth inhibitory concentration of lysine plus treonine has led to the isolation of lysine and treonine (LT) resistant mutant. Highly LT-resistant mutant line was determined as (RLT 70). About 70-80% of the wild-type enzyme activity was usually inhibited by lysine and the rest by treonine. Evidence for the existence of at least two AK isoenzymes was obtained by ion-exchange chromatography: the first one was lysine sensitive and the second one was threonine sensitive. Progeny test performed on regenerated plants demonstrated the dominant monogenic nuclear character of the mutation. These unique LT resistant mutants is being resistant to a lysine analogue S-2 aminoethyl-L-cysteine (AEC). An altered dihydrodipicolinate syntheses (DHDPS) enzyme in internal regulation of aspartate pathway which is totally desensitised to that normal lysine feedback inhibition, leads to amino acids lysine and treonine overproduction reaching 30% of the total are found in leaves againts 1.5% in the wild type. The high treonine contents encountered when analysing this LT-resistant mutant of *Nicotiana sylvestris* confirms the validity of the developed approach for improving crop quality, as well as the potential value of the mutated gene[2,3,7,10,15].

Sumaryati[4] has developed of sophisticated cellular invitro techniques based on protoplast culture combined with UV irradiation and mutagenesis with appropriate positive selection methods producing several interesting biochemical mutants that also showing resistant to salt and water stress condition in *N. plumbaginifolia*. These biochemical mutant have

characterised as stable proline overproduction. The accumulation of free proline may reach 40% of the total free amino acids. It is interesting to note that the mutants, which accumulate 10 to 15 fold more proline than the wild type on saline medium and still capable of a further increase in proline concentration in response to stress in the medium. The genetic basis concerning resistant above was showed very clear Mandelian segregation revealed that dominantly expressed resistance gene[2-4,7]. The successful of selection, characterization and clear genetic basis of the mutants has not been reported from others. The regenerated of mutant plants was grow faster and taller than wild type. According to NMR ^{13}C spectrum of leave extract explained that an increase of proline following by decrease of glutamate, thus it seems to be the proline is being produced from glutamat. The spectrum of sucrose moiety appeared only in the mutant suggested that the biochemical mutant also highly photosynthesis. Until recently there is only very few investigation where stable mutants have been characterised biochemically and genetically.

Another interesting results of clone diversities or genetic improvement in using Co-60 gamma rays was reported in Stevia rebaudiana Bertoni[16]. This plant is an important source of natural sweetening agent stevioside, a diterpene glucoside approximately 300 times better than sucrose. The results showed that 72 new mutant clones were induced with change of the length of shots, colour, size and shape of leaves. The genetic change was detected from PCR amplification products for every mutant clones and control parental. DNA genomic analysis of *Stevia* plantlets based on RAPD markers using arbitrarily chosen oligonucleotide primer resulted resolution of approximately 500 and 3100 bp for 1 and 5 times amplification. By using RFLP technique in examining mutant clones from irradiation treatment found that the wide variation observed were predominantly due to gamma-rays rather than a reflection of induction by tissue culture it self[8,16]. Most impressive was the great portion (35%) of mutants with increased leaf number and stevioside content compared with the original plants. This diversity would be important for breeder and industries in producing natural stevioside.

MOLECULAR BASIS OF DNA MUTATION

When Watson and Crick described the double-helix structure of DNA and proposed its semiconservative replication based on specific base-pairing to explain the faithful transmission of genetic information from generation to generation, they also proposed a mechanism to explain spontaneous mutations. Watson and Crick pointed out that the structures of the bases in DNA are not static. Hydrogen atoms can move from one position in a purine

or pyrimidine to another position, for example, from an amino group to a nitrogen ring. Such chemical fluctuations are called tautomeric shifts[11]. Although tautomeric shifts are rare, they may be of considerable importance in DNA metabolism since some can alter the pairing potential of the bases. The structures of DNA bases such as Adenine (A) Guanine (G) Cytosin (C) and Thymine (T), in which A always pairs with T, and G always pairs with C. mutation resulting from tautomeric shifts in the bases of DNA involved the replacement of a purine in the replacement of a pyrimidine are called transitions. Base pair substitutions involving the substitution of a purine for a pyrimidine and vice versa are called transversions. There will be four different transitions and eight different transversion are posible. A third type of point mutation involves the addition or deletion of one or a few base-pairs. Base-pairs additions and deletions are collectively referred to as frameshift mutations because they alter the reading frame of all base-pair triplet (specifying codons in mRNA and amino acids in the polypeptide gene-product) in the gene distance to that mutation. The key factor of spontaneous mutation appear to be (a) the accuracy of DNA replication machinery, (b) the efficiency of the numerous mechanism that have evolved for the repair of demaged DNA, and (c) the degree of exposure to mutagenic agents present in the environment[12].

The two major products of UV absorption by pyrimidines appear to be pyrimidine hydrates and pyrimidine dimers. Thymine dimmers appear to cause mutations indirectly in two ways. (1) Dimmers apparently disturbing the DNA double helix and interfere with accurate DNA replication. (2) Occasional errors are made during the cell processes for the repair of damaged DNA, such as DNA containing thymine dimmers. The relationship between mutation rate and UV dosage is highly variable, depending on the type of mutation, the organism, and the condition employed[11].

CONCLUSIONS

Invitro mutagenesis and selective selection in plant cell or tissue culture are easy ways to screen thousand of cells in order to create several important biochemical mutants and useful clones variation in plant. Clear simple genetic basis concerning mutation will allow us to isolated the gene of interest and introducing to other plants for breeding purposes.

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