CHAPTER 30

IN VITRO MUTAGENESIS AND MUTANT MULTIPLICATION

S. PREDIERI AND N. DI VIRGILIO

Istituto di Biometeorologia IBIMET-CNR, Area Ricerca CNR, Bologna, Italy.
E-mail: s.predieri@ibimet.cnr.it

1. INTRODUCTION

Induced mutations technique is a valuable tool not yet fully exploited in fruit breeding. Tissue culture makes it more efficient by allowing the handling of large populations and by increasing mutation induction efficiency, possibility of mutant recovery and speediness of cloning selected variants. Some vegetatively-propagated species are recalcitrant to plant regeneration, which can be a limit for the application of gene transfer biotechnology, but not for mutation induction breeding. Mutagenesis offers the possibility of altering only one or a few characters of an already first-rate cultivar, while preserving the overall characteristics. Traits induced by mutagenesis include plant size, blooming time and fruit ripening, fruit color, self-compatibility, self-thinning, and resistance to pathogens (Predieri, 2001). The combination of *in vitro* culture and mutagenesis is relatively inexpensive, simple and efficient (Ahloowalia, 1998). The availability of suitable selection methods could improve its effectiveness and potential applications. The molecular marker technology available today already provides tools to assist in mutation induction protocols by investigating both genetic variation within populations and early detection of mutants with desired traits. However, cost still represents a major limitation to their application.

Among the techniques and sources of genetic variation available for tissue culture mutation induction, physical mutagens have already shown potential for application in fruit breeding. The types of radiation suitable for mutagenesis are ultraviolet radiation (UV) and ionizing radiation (X-rays, gamma-rays, alpha and beta particles, protons, and neutrons). X-rays and gamma-rays are the most convenient and easiest types of radiation to use with regards to application methods and handling (Sanada & Amano, 1998), and have been both the most widely used ionizing radiation types and the most effective for fruit breeding purposes. Furthermore, physical mutagens

S.M. Jain and H. Häggman (eds.), Protocols for Micropropagation of Woody Trees and Fruits, 323–333.

have some technical advantages over chemical mutagens. With regards to safety and environmental issues there is no need for manipulation of hazardous substances and production of toxic residues. Physical mutagen post-treatment manipulation is simpler and allows for a more precise determination of exposure time.

This manuscript describes methodologies for *in vitro* mutation induction using physical mutagens, and in particular γ-ray technology, on fruit tissue culture.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

Explants should preferably derive from certified virus-free mother plants. This initial choice strenghthens the complete protocol, including useful mutant identification and requirements for its release as a cultivar. Cultures should be free of latent microbial contaminants, since radiation may stimulate microorganism proliferation while weakening the plants. Mutation induction should be performed on well-established *in vitro* cultures that show providing a consistent proliferation rate through subcultures. Generally, after explant establishment, 5–6 subcultures are necessary to have cultures growing at a consistent rate on the proliferation medium. Proliferation media suitable for apple, pear, and plum are presented in Table 1. Cultures are incubated at 23 ± 2°C with a 16-h photoperiod and subcultured every 3–4 weeks.

Table 1. *Culture media composition.*

Medium composition	*Apple*	*Pear*	*Plum*
Mineral Salts	MS*	MS	MS
Thiamine-HCl (μM)	1.2	1.2	1.2
Myoinositol (mM)	0.55	0.55	0.55
BAP benzyladenine (μM)	4.4	6.6	3.3
IBA indole-3-butyric acid (μM)	0.5	0.5	0.5
Sucrose (w/v)	2	2	2
Agar (w/v)	0.65	0.65	0.65
pH	5.7	5.7	5.7

*MS: (Murashige & Skoog, 1962)

When an efficient regeneration protocol from plant tissue is available, treatment can be performed on tissue before inducing regeneration. However, if a species or a cultivar is recalcitrant to regeneration, treatment can be performed directly on prolixferating shoots. Actually, when the aim of the breeding program is to maintain all the traits of a cultivar and improve only one or a few specific traits, the irradiation and propagation of *in vitro* axillary shoots may be the most adequate and easiest method. Efficient micropropagation protocols are available for nearly all species of horticultural importance. Furthermore, without the passage through undifferentiated growth, the undesired influence of somaclonal variation could be avoided (van Harten, 1998). On the other hand, when mutagens are used on undifferentiated tissues and organs, without preformed axillary buds, either prior to regeneration, or in different

stages of adventitious meristem differentiation, somaclonal variation is added to the mutagen effects. This system has the advantage that when regeneration is achieved from single cells the risk of obtaining chimaeras is reduced.

2.2. Radiosensitivity Assessment

The first step of a breeding program with physical mutagens is the assessment of treatment dose. Before executing the actual mutagenic treatment is advisable to perform a preliminary study on the specific sensitivity of the material to be used. In fact, the difference in radiosensitivity can be explained not only by inherent genetic differences between cultivars but also by physiological differences. Literature is used to provide generic information for setting an experiment for assessing specific radiosensitivity. The choice of the most suitable dose is commonly based on growth reduction as a result of the treatment. The aim of the preliminary investigation is to assess the dose that results in a 50% reduction of growth (LD50 = lethal dose 50%). Growth is determined, depending on the material tested, on the basis of the number of regenerated shoots or proliferation achieved in the first subculture after treatment, indicating apical and axillary meristems survival rate. For most micropropagated fruit trees a dose of 60 Gy is expected to be higher than LD50. Thus four doses of 0, 20, 40, or 60 Gy can provide the basis for calculating LD50 of the specific material to submit to treatment.

2.2.1. Radiosensitivity Assessment on Adventitious Buds from Leaf Tissues

To provide reliable data for LD50 calculation, thirty leaves per treatment must be subjected to irradiation and four doses tested, including the unirradiated control, on which performance 50% growth reduction is calculated. Following the regeneration protocol set up by Predieri and Fasolo (1989) for apple (*Malus pumila* L.), leaves are taken from 30-day-old cultures, and only the first three apical unfurled leaves are used. Three transverse cuts are made to the midrib and the petioles are removed. Each leaf blade is then placed with the adaxial face touching the medium (Figure 1A).

The regeneration medium, suitable for a number of apple (Fasolo & Predieri, 1990) and pear (Predieri et al., 1989) cultivars, contains MS salts (Murashige & Skoog, 1962), LS vitamins (Linsmaier & Skoog, 1965), 22.2 μM benzyladenine (BA), 1.1 μM α-naphtaleneacetic acid (NAA), 2% (w/v) sucrose, agar concentration ranges from 0.65 to 0.75% (w/v) depending on brand, pH 5.7. Leaves are placed six per Petri dish and submitted to treatment (Figure 1B).

The use of acute irradiation allows rapid treatment of the plant material. Predieri and Gatti (2003) tested total doses of 0, 10, 20, 30, or 40 Gy. Acute irradiation (42.7 Gy/min) with gamma rays from a cobalt (Co^{60}) source was provided by "Gammacell 220" (Atomic Energy Canada Limited, Ottawa, Ontario, Canada) at the Institute of Photochemistry and High Energy Radiations (ISOF—CNR, Bologna) (Figure 1C).

Figure 1. *A) Leaves are taken from 30-day-old pear cultures. After three transverse cuts are made to the midrib, leaf blades are placed with the adaxial face touching the regeneration medium. B) Petri dishes containing six leaves each are placed inside a gamma cell for treatment. C)"Gammacell 220" (Atomic Energy Canada Limited, Ottawa, Ontario, Canada) with Petri dishes inside, is closed for having plant material subjected to mutagenic treatment. D) Shoots taken from 30-day-old pear cultures are placed in a Petri dish for treatment. E) After mutagenic treatment shoots are transferred to a fresh proliferation mediu. F) Proliferation rate is determined by counting the number of shoots developed from control (left) and from irradiated shoots (right: 60 Gy).*

The number of adventitious shoots regenerated was recorded after culture in the dark on regeneration medium for 45 days. LD50 was calculated as the dose of γ-radiation that reduces the number of shoots regenerated per irradiated leaf to 50% of

unirradiated control leaves, based on linear regression (Wu et al., 1978). Supposing to have four treatments of 0, 10, 20, and 30 Gy, yielding average regenerated shoots per leaf of respectively 8.8, 6.8, 3.4, 0.2, the linear regression equation results $y = -0.2911x + 9.174$ with an $R^2 = 0.9889$ (Figure 2). The Gy dose inducing DL 50 (x) is calculated with the equation, by substituting 'y' with the value of 50% of control (0Gy) regeneration: 4.4. Calculated DL50 will be: $x = (4.4 - 9.174)/-0.2911 = 16.40$.

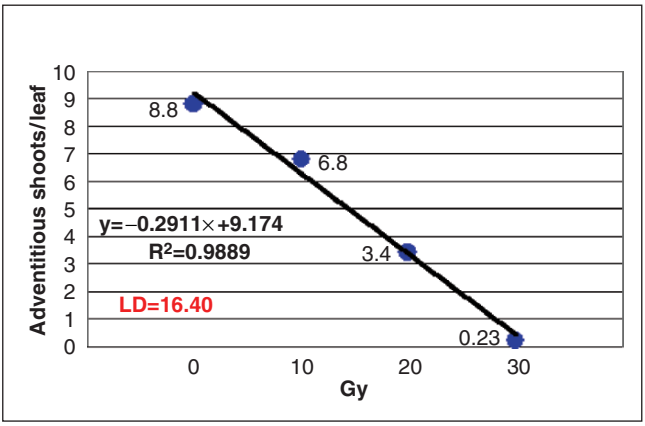

Figure 2. *Example of LD50 calculation on the regeneration response of leaves subjected to different doses of gamma ray.*

2.2.2. Radiosensitivity Assessment on Axillary Shoots from Microcuttings

When shoots are used, the protocol is aimed to support their growth after treatment and to induce the maximum proliferation of axillary buds. Microcuttings 1.5–2 cm long are cut from 30-day-old proliferating cultures and placed horizontally in plastic Petri dishes (10 cm diameter) with a few drops of sterile water added to protect shoots from dehydration during treatment (Figure 1D). After irradiation, shoots are transferred to jars containing proliferation medium (Figure 1E). Proliferation rate is recorded after 30 days of culture (Figure 1F). LD50 is calculated as the dose of γ-radiation that reduces the proliferation rate of irradiated shoots to 50% of unirradiated control shoots, based on linear regression (Wu et al., 1978), as described for regeneration in 2.2.1.

2.3. Treatment

Cultures are prepared for treatment following the protocol described for radiosensitivity determination. After the estimation of the dose inducing LD50, the most convenient dose for treatment can be chosen. The actual dose to be applied in a particular breeding project is chosen based on the breeder's experience with the specific plant material, its genetics, and its physiology, with the aim of having the highest probability of useful mutant rescue. Heinze and Schmidt (1995) suggested as

a starting point for the experimental protocol doses giving LD50 ± 10%). Doses lower than LD50 favour plant recovery after treatment, while the use of higher doses increases the probability to induce mutations (either positive or negative).

Once treatment dose is chosen, more information is needed to decide how many shoots or leaves to submit to treatment. The number of plants (P) to be obtained is calculated on the basis of the expected frequency of induction of the desired trait. Expected mutations frequencies for single trait can be expected to appear with a frequency of 0.1–1.0%. Predieri and Zimmerman (2001) report for a number of variation in fruit traits in different cultivars of pear, frequencies ranging form 0.14 to 1.93%.

To stay on the safe side, we set an expected frequency of 0.5%, out of 1000 plants 5 individuals would be carrying the desired trait. The breeder's experience and a thumb rule that must also take in account economic costs should be applied. However, plan to work with less than 500 plants limits the possiblity of successful selection. Eight hundred or better thousand plants (P) appears to be the minimum to provide reasonable opportunities for selection.

Some basic information is also needed: a) expected regeneration/proliferation rate of the irradiated material; b) number of subcultures after treatment before rooting microcuttings; c) expected percent shoot rooting; d) expected percent plant survival. Expected proliferation rate should be calculated with the same regression equation used for calculating LD50. It presumably will increase in the subcultures following the first, but it is advisable to stay on the safe side, and plan to produce more plants to face unexpected contamination, rooting or survival problems.

The number of subcultures needed after treatment vary from a minimum of 3 to a maximum of 5 depending of the care exercised on avoiding chimaeras. Rooting percentage can be calculated to be about 80% of the regular frequency obtained for the material or more directly obtained by observing cultures treated for LD determination. Plant survival must be calculated on the basis of experience with the specific plant material.

To calculate how many shoots (X) to submit to treatment use the following formula:

$$X = P/((a * b)*c)*d.$$

e.g. Number of plants planned for field selection: 1000. a) expected proliferation rate 3.3; b) number of subcultures 4; c) expected rooting percent 0.85; d) expected plant survival 0.90. X = 1000/((3.3*4)*0.85)*0.90 = 99.

2.4. Post-treatment Handling

2.4.1. Post-treatment Care

The use of acute irradiation allows rapid treatment of the plant material, less than 1 min for reaching the LD50 in the cases described in Predieri and Gatti (2003). The irradiation of a high number of meristems in easy to manipulate and transport vessels, such as Petri dishes, allows to save space and to use even small irradiation

facilities (e.g. gamma cells), thus facilitating also the execution of the required post-irradiation handling. Undesired negative primary effects of radiations, causing tissue browning, necrosis or chlorosis, are unavoidable. However, appropriate handling of plant material can provide the desirable limitation of physiological effects that favors mutant survival and the emergence of phenotypic variation determined by genetic changes. Cultures irradiated either during proliferation or regeneration should be transferred rapidly to a fresh medium, to avoid the formation of toxic compounds (Ahloowalia, 1998). The basal part of irradiated shoots (1 mm) should also be removed for the same reason. Avoiding to exposure of the culture to high light intensity in the first hours after treatment may also help in limiting negative physiological effects induced by irradiation.

2.4.2. Irradiated Shoot Proliferation

In vitro techniques allow for the rapid execution of propagation cycles of subculture aimed to separate mutated from non-mutated sectors (Ahloowalia, 1998). After transfer to fresh regeneration medium, irradiated tissues can be subjected to standard regeneration procedures, since their aim is already to maximize shoot differentiation and growth (Figure 3A).

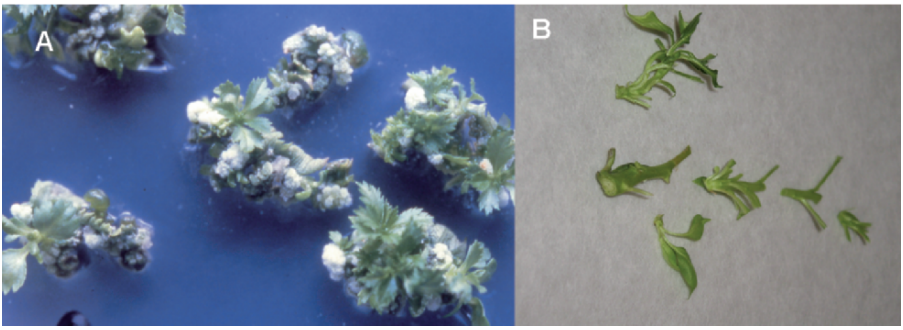

Figure 3. *A) Shoots are grown on the regeneration medium until developed enough (2–3 mm) to be transferred to proliferation medium. B) At each subculture propagules should be separated in 1–2 nodes cuttings to stimulate axillary bud development.*

However, when microcuttings already provided of meristems are irradiated, after treatment culture should be aimed to help all treated meristems to develop shoots. This is done through repeated cuttings to avoid apical dominance effects (Figure 3B), coupled with a convenient choice of growth regulators to support shoot growth. Cytokinins can be reduced to one half of the standard concentration for the first subculture after treatment, and increased to the full concentration for the subsequent subcultures.

Following mutagen treatments, cultures are chimaeras, composed of non-mutated cells and cells carrying different mutations. To avoid chimaeras is important to guarantee that all the treated shoots undergo at least three subcultures. The protocol

proposed by Predieri (2001) (Figure 4) can be adopted and adapted to the requirements of the different species of interest.

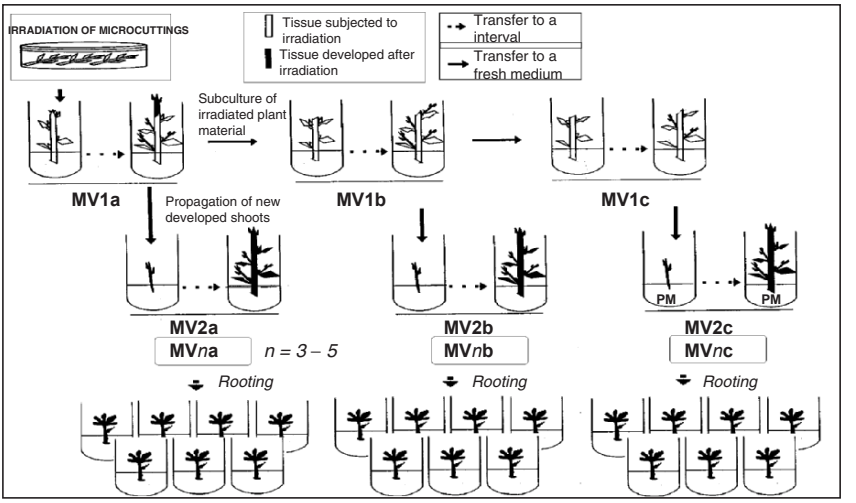

Figure 4. *Protocol for post-irradiation culture multiplication (modified from Predieri, 2001).*

2.4.3. Rooting

Undesired physiological effects can be observed even at the rooting stage, where they negatively affect the microcutting's rooting capacity (Jain, 1997; Predieri & Gatti, 2000). Particular care should be exercised at this stage and during the following acclimation to prevent the loss of useful mutants due to impared rooting ability. For a number of species a short, acute, auxin treatment (2–8 days) followed by transfer to auxin-free medium can help in increasing rooting as compared to performances achieved by maintaining microcuttings for all the rooting period (4–5 weeks) on the same rooting medium.

2.5. Selection

2.5.1. In Vitro Selection

The identification and selection of desired mutants should be performed on non-chimaeric plants. As compared to methodologies involving treatment of *in vivo* buds, *in vitro* culture provides a wider choice of plant material composed of a few or even just one cell for mutagenic treament (Maluszynsky et al., 1995). This results in a lesser risk of obtaining chimaeric plants and an higher probability of obtaining homohistonts having mutated cells expressing the mutation in the phenotype. When effective selection methods are available, they can be used directly *in vitro* on the population obtained from propagation performed after mutagenic treatment. Several screening methods have been developed with regards to resistance to pathogens,

tolerance to high pH or elevated metal concentration, and selection of reduced vigor individuals (Predieri, 2001). Screening performed *in vitro* allows for handling of large populations, avoiding the problem of working with a low number of inviduals. Different selection pressures can be applied, e.g. to reduce population to 10%. *In vitro* selected variants should always undergo specific *ex-vitro* testing to confirm the existence of improved selected traits and to exclude the possible emergence of other undesired traits.

2.5.2. Ex Vitro Selection

When *in vitro* selection methods are not available, mutant identification must be postponed to field observation. Predieri et al. (1997) set up a protocol for the selection of compact pears in a population subjected to mutagenic treatment. Given the impossible burden of measuring thousands of plants, three groups were defined representing about 1/10 of the whole population: 1) control, which consisted of non-irradiated, micropropagated plants; 2) sample, which consisted of every tenth plant in the row; 3) selected, which consisted of trees chosen because they exhibited variation in vegetative growth characteristics. On these plants phenotypic data were collected on shoot length and diameter and number of nodes, spurs and lateral shoots, plant height and trunk diameter. Cluster analysis was performed on data to select trees carrying interesting traits. As related to pomological traits, data were collected for selecting: a) early bearing small trees; b) high productivity combined with a high production efficiency; c) consistent production and high fruit weight.

2.6. Advances in Mutant Identification

The range of methods available for the identification of mutations is widening (Ahloowalia & Maluszynski, 2001). However, none of the techniques can guarantee the identification of mutants carrying a single random mutation in the genome. Two methods showing potential application have been discussed by Karp (2000). These methods are based on the detection of changes known to be induced at high frequency in tissue culture: (1) AFLPs with methylation-sensitive enzymes and (2) detection transposon insertional polymorphisms. When markers linked to traits of interest are available, a marker-assisted selection can be performed. For this purpose, highly saturated linkage maps can provide a choice of markers closely linked to a specific trait. An increasing number of markers linked to agronomically important traits are now being identified, providing even more opportunities for marker-assisted selection. Recently developed technologies such as TILLING (Targeting Induced Local Lesions IN Genomes) (Slade & Knauf, 2005) appear to provide new opportunities for early mutant identification. Another promising technique is S-SAP (sequence-specific amplified polymorphism), which has been applied to study genetic bases of bud mutations in apple (Venturi et al., 2006).

3. CONCLUDING REMARKS

Mutation induction treatments performed on *in vitro* shoots or on plant tissues prior to regeneration have a potential for contributing to fruit breeding, allowing for single-trait changes in the improvement of a cultivar, while retaining yield parameters. Induced mutagenesis with the use of physical mutagens appears to be a ready-to-use technique, since a large number of fruits would benefit from the improvement of single specific key traits. The interest in results of mutations breeding is demonstrated the impact of mutation derived varieties (Ahloowalia et al., 2004) and by the continuing interest in "clones" of fruit cultivars (Sturm et al., 2003; Venturi et al., 2006), often simply selected by chance in the field, which have generated new patented varieties. The first steps of such a protocol are easy and relatively cheap. *In vitro* mutation induction results could be increased by the use of effective early screening methods for biotic agents and abiotic factors. However, only the planning of extensive field trials could allow a real evaluation of selected mutants' potential.

The molecular approach offers tools for increased understanding of specific DNA changes caused by mutagenic treatments. This can be of great help in reducing the unpredictability of the results of breeding protocols based on nuclear technology. Jain (2005) recommends the development of a molecular database as an instrument for predicting expected mutations. Mutation-assisted breeding programs can become a reliable method for tropical and subtropical fruit crop improvement in countries where enhanced food production and sustainability are of great importance. However, often tissue culture can be applied only if it is cost-effective. The research of low-cost procedures for tissue-cultured plant production is crucial for the development of new opportunities of exploitation. Research efforts should be focused also into the development of new regeneration and selection methods, e.g. those based on cell suspension culture (Ahloowalia & Maluszynski, 2001). Tissue culture, supported by expertise on genetics, plant pathology, and molecular biology can effectively use induced mutation procedures as a tool in plant improvement. Every advance in the understanding of *in vitro* plant physiology and the improvement of tissue culture efficiency can open new opportunities for the development of successful mutation breeding programs.

Acknowledgments. Authors wish to thank Dr. A.E. Sztein for the revision of the manuscript.

4. REFERENCES

Ahloowalia, B.S. (1998) *In-vitro* techniques and mutagenesis for the improvement of vegetatively propagated plants. In: S.M. Jain, D.S. Brar & B.S. Ahloowalia (Eds) *Somaclonal Variation and Induced Mutations in Crop Improvement* (pp. 293–309). Kluwer Acad. Pub., Dordrecht.

Ahloowalia, B.S. & Maluszynski, M. (2001) Induced mutations – A new paradigm in plant breeding. Euphytica 118, 167–173.

Ahloowalia, B.S., Maluszynski, M. & Nichterlein, K. (2004) Global impact of mutation-derived varieties. Euphytica 135, 187–204.

Fasolo, F. & Predieri, S. (1990) Cultivar dependent responses to regeneration from leaves in apple. Acta Hort. 280, 61–68.

Heinze, B. & Schmidt, J. (1995) Mutation work with somatic embryogenesis in woody plants. In: S.M. Jain, K. Gupta & J. Newton (Eds) *Somatic Embryogenesis in Woody Plants*, Vol 1 (pp 379–398). Kluwer Acad. Pub., Dordrecht.

Jain, S.M. (1997) Creation of variability by mutation and tissue culture for improving plants. Acta Hort. 447, 69–77.

Jain, S.M. (2005) Major mutation assisted plant breeding programs supported by FAO/IAEA. Plant Cell Tiss. Org. Cult. 82, 113–123.

Karp, A. (2000) Molecular tools for detecting genetic diversity. Acta Hort. 530, 17–32.

Linsmaier, E.M. & Skoog, F. (1965) Organic growth requirements of tobacco tissue culture. Physiol. Plant. 18, 100–127.

Murashige, T. & Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 241–251.

Predieri, S. (2001) Induced mutation and tissue culture in fruits. Plant Cell Tiss. Org. Cult. 64, 185–210.

Predieri, S. & Fasolo, F. (1989) High-frequency shoot regeneration from leaves of the apple rootstock M26 (Malus pumila Mill.). Plant Cell Tiss. Org. Cult. 17, 133–142.

Predieri, S. & Gatti, E. (2000**)** Effects of gamma radiation on microcuttings of plum (*Prunus salicina* Lindl.) 'Shiro'. Adv. in Hort. Sci. 14, 7–11.

Predieri, S. & Gatti, E. (2003) In vitro techniques and physical mutagens for the improvement of fruit crops. In: A. Mujib, et al. Eds *In vitro Applications in Crop Improvement: Recent Progress* (pp. 20–34). Science Pub. Inc.

Predieri, S. & Zimmerman, R.H. (2001). Pear mutagenesis: In vitro treatment with gamma-rays and field selection for productivity and fruit traits. Euphytica 3, 217–227.

Predieri, S., Fasolo, F., James, D.J., Passey, A.J. & Ridout, M.S. (1989) Regeneration from *in vitro* leaves of 'Conference' and other pear cultivars (*Pyrus communis* L.). J. Hort. Sci. 64, 553–559.

Predieri, S., Magli, M. & Zimmerman, R.H. (1997). Pear mutagenesis: In vitro treatment with gamma-rays and field selection for vegetative traits. Euphytica 93, 227–237.

Sanada, T. & Amano, E. (1998) Induced mutation in fruit trees. In: S.M. Jain, D.S. Brar & B.S. Ahloowalia (Eds) *Somaclonal Variation and Induced Mutations in Crop Improvement* (pp. 401–409). Kluwer Acad. Pub., Dordrecht.

Slade, A.J. & Knauf, V.C. (2005) Tilling moves beyond functional genomics into crop improvement. J. Transgenic Res. 14, 109–115.

Sturm, K., Hudina, M., Solar, A., Marn, M.V. & Stampar, F. (2003) Fruit quality of different 'Gala' clones. Eur. J. Hort. Sci. 68, 169−175.

Venturi, S., Dondini, L., Donini, P. & Sansavini, S. (2006) Retrotransposon characterisation and fingerprinting of apple clones by S-SAP markers. Theor. Appl. Gen. 112, 440–444.

Wu, F.F., Siddiqui, S.H., Heinz, D.J. & Ladds, S.L. (1978) Evaluation of mathematical methods for predicting optimum dose of gamma radiation in sugar cane. Environ. Exp. Bot. 18, 95–98.