C. Broertjes

Use in plant breeding of acute, chronic or fractionated doses of X-rays or fast neutrons as illustrated with leaves of Saintpaulia

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de Rector Magnificus, Mr. J.M. Polak,
hoogleraar in de rechts- en staatswetenschappen
van de westerse gebieden,
in het openbaar te verdedigen
in de aula van de Landbouwhogeschool te Wageningen
op vrijdag 23 juni 1971 te 16 uur

1972 *Centre for Agricultural Publishing and Documentation*

Wageningen

Facta canam, sed erunt qui me finxisse loquantur.

Ovidius, Fasti VI, 3.

(I'll state the facts, but some will say I have imagined things).

(Ik ga ware gebeurtenissen vertellen; maar sommigen zullen zeggen, dat ik fantaseer).

Dit proefschrift met stellingen van Cornelis Broertjes, landbouwkundig ingenieur, geboren te Haarlem op 31 maart 1923, is goedgekeurd door de promotor, dr. ir. J. Sneep, hoogleraar in de leer van de plantenveredeling.

De Rector Magnificus van de Landbouwhogeschool
J.M. Polak

Wageningen, 3 mei 1972

ISBN 90 220 0389 2

This thesis will also be published as Agricultural Research Reports 776.

Stellingen - Assertions

1.

Het verdient aanbeveling om bepaalde gefractioneerde bestralingen, die bij *Saintpaulia* een duidelijke verhoging van het aantal mutanten per 100 bestraalde bladeren tot gevolg hebben, nader op hun waarde te toetsen bij minder heterozygote vegetatief vermeerderde gewassen.

It is recommendable to investigate whether given fractionated irradiations, which, in *Saintpaulia*, result in an evidently increased number of mutants per 100 irradiated leaves, also do so with less heterozygous vegetatively propagated crops.

Dit proefschrift, p. 64.

2.

De adventief-spruit-methode, waarmede, in vergelijking met bestraling van plantedelen met multicellulaire vegetatiepunten, meer en volledige (niet-chimaere) mutanten verkregen kunnen worden na een mutagene behandeling van afgesneden bladeren, is een belangrijk hulpmiddel bij de veredeling van vegetatief vermeerderde gewassen.

The adventitious bud technique, resulting in more and solid, non-chimeral mutants after a mutagenic treatment of detached leaves, is a better method of breeding vegetatively propagated crops than the treatment of plant parts with multicellular buds.

Dit proefschrift, p. 18.

3.

De resultaten van vele in de literatuur beschreven experimenten met gefractioneerde bestraling zijn moeilijk te interpreteren en hebben een beperkte wetenschappelijke waarde omdat met niet of onvolledig gesynchroniseerde celpopulaties is gewerkt terwijl bovendien onvolledige reeksen (initiële) doses en

tijd-intervallen zijn toegepast.

The results of many published experiments, describing dose fractionation, are difficult to interprete and have a restricted scientific value since incompletely synchronized or asynchronous cell populations have been used and moreover incomplete series of (initial) doses and time intervals have been applied.

Bijvoorbeeld:
D.R. Davies, 1962. Strahlenwirkung und Milieu: 160-170.
R.J. Horsley and A. Laszlo, 1971. Int. J. Radiat. Biol. 20: 593-596.
W. Sheridan, 1971. Mut. Res. 13: 163-169.

4.

Uit hun conclusie, dat bij gelijk niveau van M_2-fertiliteit EMS 2 à 3x zoveel bloeitijd- en chlorophyl mutaties induceert als röntgenstralen, mag niet zonder meer worden afgeleid, dat dit ook zou gelden voor al die mutaties, die van belang zijn in de praktische mutatieveredeling.

From their conclusion, that at equal levels of M_2-fertility EMS produces 2-3 times more flowering time and chlorophyl mutations than X-rays, it does not follow that this also applies to all mutations that are important in practical mutation breeding.

H.A.S. Hussein, 1968. Genetic analysis of mutagen-induced flowering time variation in *Arabidopsis thaliana* (L) Heynh. Proefschrift (H. Veenman en Zn, N.V., Wageningen).
M. Mesken and J.H. van der Veen, 1968. The problem of induced sterility: a comparison between EMS and X-rays in *Arabidopsis thaliana*. Euphytica 17: 363-370.

5.

Er wordt onvoldoende rekening gehouden met het feit, dat insektenbestrijding met behulp van de "sterile insect release method" onherroepelijk tot teleurstellende resultaten leidt zonder grondige en vooral kwantitatieve veldbiologische kennis van de betreffende plaag.

Without deep knowledge, in particular quantitative, of the ecology of an insect species, the "sterile insect release method" will fail.

Insect ecology and the sterile male technique. Proc. FAO/IAEA, Vienna, 1969.

6.

Het toevoegen van bepaalde chemische stoffen aan voedsel ter verbetering van houdbaarheid en kwaliteit dient uit het oogpunt van volksgezondheid onverwijld te worden gestaakt en vervangen te worden door onschadelijke fysische methoden zoals bestraling.

The addition of certain chemicals to food to improve the keepability and quality should be stopped immediately in the interest of public health and replaced by physical methods, such as radiation.

7.

De hypothese dat er specifieke orgaan-vormende stoffen in de plant bestaan is onjuist.

The hypothesis that specific organ-forming substances exist in plants is not true.

E.W. Sinnott, 1960. Plant morphogenesis (p.413; p.376). Mc.Graw-Hill Book Co. N.York-Toronto-London.

8.

Het anoniem beoordelen van met een auteursnaam voorziene wetenschappelijke manuscripten, voordat deze (al of niet) worden gepubliceerd in een tijdschrift, is ontoelaatbaar.

Reviewing or refereeing of signed scientific articles as a condition for acceptance should not be anonymous.

9.

De door Westhoff geuite veronderstelling, dat het aantal potentieel waardevolle kamerplanten veel groter is dan dat wat tot nu toe zijn weg naar het publiek gevonden heeft, moet worden betwijfeld.

Westhoff's supposition that the number of potentially valuable pot plants greatly exceeds the number so far introduced to the public, seems doubtful.

V.W. Westhoff, 1971. De botanische tuin in de samenleving, p.18 (Rede ter gelegenheid van de officiële opening van de Botanische Tuin Nijmegen).

10.

Het gebruiken van hoogtepunten uit de klassieke muziek bij TV-reclame moest uit cultureel-hygiënische overwegingen worden verboden. Het roept ongewilde associaties op met wasmiddelen en deodorants bij de concertbezoeker.

The use of the best of classical music in TV commercials should be purged since at concerts it conjures up unwanted associations with detergents and deodorants.

Proefschrift van C. Broertjes
Wageningen, 23 juni 1972.

Abstract

Broertjes, C. (1972) Use in plant breeding of acute, chronic or fractionated doses of X-rays or fast neutrons as illustrated with leaves of *Saintpaulia*. Doctoral thesis, Wageningen. ISBN 90 220 0389 2, (viii) + 74 p., 9 tbs, 31 figs, 8 photographs, 134 refs, Eng. and Dutch summaries. *Also* Agric. Res. Rep. (Versl. landbouwk. Onderz.) 776.

The parameters used were survival of irradiated leaves of *S. ionantha*, production of adventitious plantlets at the base of the petiole and mutation frequency.

The differences between unfiltered X-rays from a 250/25 deep therapy apparatus and fast neutrons from a degraded fissions spectrum was slight and sometimes negligible. A striking dose rate effect was observed, especially with X-rays. In fractionation experiments a protective mechanism was induced in the leaves by pre-irradiation with either type of radiation. This mechanism reached a maximum after 8-12 h. Then the extent of the protection was equivalent to 3 krad X-rays or 1.5 krad fast neutrons after a single pre-treatment with the optimum initial dose of 500 rad, X-rays or fast neutrons. A 10-15 fold irradiation with this dose, separated by 8-h intervals, induced a protection of 6-7 krad X-rays or 3-4 krad fast neutrons.

Several of the treatments result in a higher number of mutants per 100 irradiated leaves compared with the optimum acute dose of 3 krad X-rays or 1.5 krad fast neutrons.

Samenvatting

Afgesneden bladeren van het Kaaps Viooltje, *Saintpaulia ionantha* H. Wendl. cv. Utrecht, werden acuut, chronisch of gefractioneerd behandeld met X-stralen of snelle neutronen. Het doel was om de optimale behandelingswijze vast te stellen voor het verkrijgen van mutanten ten behoeve van de plantenveredeling.

Het effect van de verschillende behandelingen werd uitgedrukt in

1. aantal overlevende bladeren, in % van de controle,
2. aantal adventiefplantjes aan de voet van de bladsteel, per blad en in % van de controle (produktie), en
3. mutatiefrequentie en aantal mutanten per 100 bestraalde bladeren.

De adventiefplantjes vertonen twee eigenschappen, die ze bijzonder bruikbaar maken voor dit onderzoek. In de eerste plaats omdat de cellen, die later betrokken zijn bij de vorming van adventiefplantjes, zich gedurende de gehele behandeling niet delen. In de tweede plaats omdat het vegetatiepunt van een adventiefplantje uiteindelijk afkomstig is van slechts één epidermiscel. Dit blijkt uit het feit, dat de gevonden mutanten, behoudens enkele uitzonderingen, een niet-chimaere structuur vertoonden en dat sectoriaalchimaeren niet werden aangetroffen.

Uit de resultaten bleek een sterk effect van doseringssnelheid op overleving, produktie en mutatiefrequentie. Na 2 rad/min was de LD_{50} voor overleving ongeveer 100 krad X-stralen, terwijl de LD_{50} bij 200 rad/min ongeveer 5 krad bedroeg. De "kritische doseringssnelheid", waarbij een maximaal effect wordt verkregen bij een verandering in doseringssnelheid, bleek ongeveer 7 rad/min te zijn (voor X-stralen).

Experimenten, waarbij de totale dosis in fracties gescheiden door één of meer tijdintervallen werd gegeven, toonden aan, dat een betrekkelijk lage initiële dosis een mechanisme induceert, dat de bladeren gedeeltelijk beschermt tegen een of meer volgende stralingsdoses. De optimale initiële dosis die maximale bescherming veroorzaakt bleek 500 rad te zijn, zowel bij X-stralen als snelle neutronen. Het optimale tijdinterval waarna de bescherming een maximum heeft bereikt bleek 8-12 uur te zijn, echter afhankelijk van de grootte van de initiële dosis en die van de volgende dosis (doses). De grootte van de bescher-

ming bleek equivalent te zijn met 3-4 krad X-stralen of 1.5-2 krad snelle neutronen, na een eenmalige voorbehandeling met de optimale initiële dosis. De grootte van de bescherming werd echter ongeveer verdubbeld na een 10-15-voudige voorbehandeling met de optimale initiële dosis, gescheiden door de optimale tijdinterval van 8 uur. Een acute dosis van bijvoorbeeld 6.5 krad X-stralen was dodelijk. Na een voorbehandeling met 500 rad gaf diezelfde dosis 100% overleving (zie de 6 krad X-lijn in Fig. 12), terwijl een tweede dosis van 10 krad nog 25% overleving gaf. Na 5 voorbehandelingen met 500 rad X-stralen, gescheiden door 8-urige intervallen, werd echter weer 100% overleving verkregen na een laatste dosis van 10 krad.

Overleving, produktie zowel als aantal mutanten reageerden over het algemeen op gelijke wijze op diverse behandelingswijzen. Daar de eerste twee parameters veel sterker reageerden op een variatie van diverse factoren dan de derde parameter (mutatiefrequentie), bleek het mogelijk te zijn om optimale behandelingsmethoden te berekenen.

Uitgaande van het aantal mutanten per 100 bestraalde blaadjes, uitgedrukt in de overeenkomstige optimale acute dosis (3 krad X-stralen of 1.5 krad snelle neutronen) werd aangetoond, dat grotere aantallen mutanten verkregen kunnen worden na bepaalde doseringssnelheden of gefractioneerde bestralingen dan na een optimale acute bestraling. Voorbeelden zijn een voorbehandeling met 500 rad, een tweede dosis van 6 krad, gescheiden door een interval van 8 uur met X-stralen of een soortgelijke behandeling met snelle neutronen (zie Tabel 9). Andere behandelingen zijn niet praktisch of te kostbaar.

Het verschil in effect tussen X-stralen en snelle neutronen (met een gemiddelde energie van 1.7 MeV) bleek verrassend klein te zijn en het is dan ook moeilijk aan te geven welke straling de voorkeur verdient.

De resultaten beschreven in dit rapport hebben uitsluitend betrekking op *Saintpaulia*. Bij andere gewassen, die mogelijk minder heterozygoot zijn en dus veel minder mutaties produceren, zou een verhoging van de mutatiefrequentie of een verschuiving in het mutatiespectrum wèl duidelijker en van meer betekenis kunnen zijn.

Het belang van de adventiefspruit-methode voor de mutatieveredeling in het algemeen is speciaal besproken met het oog op de rol van de diplontische selectie. Er zijn redenen aangevoerd ter ondersteuning van de veronderstelling, dat de diplontische selectie in normale multicellulaire vegetatiepunten groter is dan bij het gebruik van de adventiefspruit-methode.

Curriculum vitae

De auteur behaalde in 1941 het einddiploma van de toenmalige 2e HBS-B te Haarlem. Vervolgens studeerde hij tropische plantenteelt aan de Landbouwhogeschool te Wageningen, met als bijvakken plantenveredeling en erfelijkheidsleer.

Na het behalen van het ingenieursdiploma in 1949 was hij een tiental jaren werkzaam als plantenveredelaar op het Proefstation voor de Boomkwekerij te Boskoop, aldaar gedetacheerd door het Instituut voor Veredeling van Tuinbouwgewassen te Wageningen.

In 1959 trad hij in dienst van het Instituut voor Atoomenergie in de Landbouw te Wageningen, dat hem van 1959 tot medio 1960 naar het Brookhaven National Laboratory op Long Island, U.S.A., zond om zich te specialiseren in de mutatieveredeling.

Contents

1 Introduction

The objective of mutation breeding is to obtain the highest possible frequency of mutants and the best possible mutation spectrum in a given crop. It enables the plant breeder, on an economically justified basis, to select for useful or improved genotypes without exceeding certain limits, such as number of plants to be handled, space and time needed and total costs.

To achieve this objective many factors have to be considered. Three of them are discussed in section 1.1:

1. the kind of mutagen,
2. the type of mutational events desired, and
3. the problems, related to the choice of the material to be treated.

It is postulated, in Section 1.1, that the nature of the mutational event depends on the radiation type (X-rays, fast neutrons) as well as on the method of irradiation (acute, chronic, fractionated). Both are discussed in Section 1.2.

Moreover it is made clear that the kind of plant material treated is a decisive factor in the mutation frequency, the mutation spectrum as well as in the ease with which mutational events can be recovered. This recovery refers to the competition within a multicellular apex (diplontic selection: Section 1.3) and the adventitious bud technique used (the apex of the buds originates from a single epidermal cell: Section 1.4).

Finally it is thought that radiation type and method of irradiation may possibly be manipulated in such a way that the repair of the radiation damage is optimum (minimum physiological effect) and includes a reasonable quality and quantity of mutational events. This topic is covered in Chapter 3 (Experiments) after Chapter 2 (Material and methods).

1.1 FACTORS OF IMPORTANCE FOR MUTATION BREEDING

To realize the objective of mutation breeding a number of factors have to be considered, such as the kind of mutagen, the type of mutational event desired and the problems, related to the choice of plant material to be

irradiated.

The final decision about the kind of mutagen depends upon the crop involved and the plant part to be irradiated. In plants propagated by seeds that are usually treated, both chemicals and ionizing radiation are effective mutagens (Gaul, 1963). In vegetatively propagated species when growing plants or plant parts have to be treated, the more penetrating types of ionizing radiation (X-rays, γ-rays, fast neutrons) are better. Chemicals generally give poor results with vegetatively propagated material because the plant parts take up much less solution when soaked than seeds. Higher concentrations do not compensate for this deficiency because they have harmful side-effects (Bowen, 1965; Nybom & Koch, 1965). When using chemicals, the type (base analogues - generally, but not exclusively used in studies with micro-organisms - alkylating agents and other mutagenic compounds), concentration, duration, temperature, modifying factors (pH, metallic ions, carrier agents and after-effects) as well as post-treatments (washing) have to be considered (Heslot, Kamra, Brunner, Konzak, Narayanan, Wickham and de Kock in: Manual on mutation breeding; IAEA Techn. Report 119 (1970): 53-77). But even when ample solution is taken up under standardized conditions (leaves of African Violet, treated with ethyl methane sulphonate), the mutagenic effect was poor compared with ionizing radiation.

The type of mutational event, in other words the mutation spectrum, very much depends on the mutagen used. Different mutation spectra can be distinguished by different ratios between gene mutations, small deficiences and chromosome aberrations. Densely ionizing radiations, like neutrons, produce more aberrations whereas X or gamma rays induce more gene mutations. Likewise, certain chemicals, such as 8-ethoxycaffeine, produce more chromosome breaks and resulting aberrations than ethyl methane sulphonate. But the dose and modifying factors, which influence the radiosensitivity (such as oxygen), also change the mutation spectrum (Micke, 1970; Nilan, 1966; Smith, 1961).

Radiation has a very complex effect upon living matter. The energy, dissipated in the cells through ionization, is the starting point of a number of chain reactions that affect all kinds of cell constituents, such as cell membranes (rupture of plasma membranes, disorganization of internal mitochondria membranes or more subtile effects), which are important to maintain cell integrity and viability. Radiation also has an effect on energy metabolism, reduces the phosphorylation, interferes with the enzyme activity and the rate of DNA synthesis. The nucleus is also a major site of radiation damage.

The precise relationship between such cytoplasmic and nuclear factors is not clear, since the final end-point is also modified by exposure conditions, cell activity during exposure, type of cell and many other circumstances (Bacq & Alexander, 1961; Casarett, 1968; Evans, 1967b).

Part of the damage is restored by repair mechanisms. These repair mechanisms - in bacteria there are at least three, but probably more - seem to be linked to particular enzyme activities with distinct repair time classes, namely < 2 min, < 15 min, and > 30 min (Kaplan, 1971). Several kinds of restoration can be distinguished, such as the neutralization of the primary effects and that of the secondary effects (resynthesis of molecules destroyed or inactivated by the irradiation). In addition to such intracellular recovery, intercellular recovery takes place, by which actively dividing cells replace, after a latent period, the dead ones (Bacq & Alexander, 1961).

The final effect, after the repair of sublethal and reversible damage, is called the permanent physiological and genetic effect and can be measured in different ways: the survival of plants or plant parts, the height of seedlings, the length of plant parts or the dry weight of the plant after a given period. Biochemical parameters have also been used, such as changes in enzyme activities (Bacq & Alexander, 1961) or in different types of DNA damage (Ebert & Howard, 1963), including different types of mutational events (gene mutations, chromosome rearrangements, reduced fertility).

In this study detached leaves of African Violet, which develop adventitious plantlets at the base of the petiole after rooting, were used to investigate the effect of radiation (see Section 1.4). Two physiological parameters were used to measure the effects (number of surviving leaves, hereafter called "survival" and number of adventitious plantlets per leaf, hereafter called "production") and one genetic parameter (number of visible mutations) (see Section 2.3).

These parameters were not only selected to express radiation effects, but mainly because they are of essential importance to the practical breeder, whose first question is how to obtain (the highest number of) useful mutants.

The physiological parameters will be referred to as the "physiological effect", whereas the number of mutations will be called "genetic effect" (see Section 3.1.1). It is realized, however, that this distinction is somewhat artificial because part of the physiological effect is determined by genetic elements (e.g. gross chromosome rearrangements as a result of chromosome breakage reduce fertility as well as vitality).

The physiological effect depends upon a great number of factors, such as

the radiosensitivity of the plant species, total dose, dose rate, type of radiation and a number of climate and other environmental conditions before, during and after the treatment (Briggs, 1968; Gaul et al., 1966; Nayaranan & Konzak, 1969).

The genetic effect upon a given genotype depends on type of radiation, dose and dose rate, but the role of environmental conditions is much less obvious and for the greater part unknown. Moreover, it depends greatly on the genotype involved; in heterozygous diploid plants the mutations, which are usually recessive, may manifest themselves immediately in the double recessive form in the irradiated material itself. When homozygous characters are involved, a second generation is needed after self-fertilizing M_1 before mutations can be scored. In polyploid crops (potato, 4 n), *Chrysanthemum* (6 n), *Dahlia* (8 n)) very complex situations may occur when different genes are present in different ploidy-levels (from multiplex to quadruplex in a tetraploid, for instance). Moreover, polyploids are less radiosensitive (Sparrow, 1962). These situations result in an obscure relationship between the treatment and the result.

Both physiological and genetic effects can thus be modified at will, relatively speaking. Since repair of physiological and genetic effects *a priori* may be the result of (partly) different processes, it might be possible to manipulate the two effects by a proper treatment so that the physiological damage (reduced survival, stunted growth, reduced fertility) as well as the undesirable part of the genetic effect (chromosome aberrations; also effecting the physiological parameters mentioned before) is minimum whereas the desired genetic effect (beneficial mutations, favourable chromosome rearrangements) is maximum. It would indeed be of great importance to the plant breeder if clear-cut methods were available to produce the optimum number of useful mutants.

In relation to chimera formation and diplontic selection (see Sections 1.4 and 1.3, respectively), the choice of the plant part to be treated is another important aspect of applied mutation breeding. Generally plant material with multicellular apices are irradiated, such as seeds, tubers, bulbs, rhizomes, stolons, scions, cuttings, etc. unless gametes (pollen or eggcells) or zygotes in the earliest developmental stages are being used.

Since a mutation is a unicellular event, the mutated cell will be exposed to a competition process in such a multicellular apex. This competition between the mutated cell and the surrounding non-mutated cells is often lost by the mutated cell as a result of the general competition between cells in different

regions with different degrees of activity. Possible accompanying damage and the subsequent reduced growth rate may also go against the mutated cell in the competitive struggle. When this takes place during haplophase it is called "haplontic selection", and when it takes place during diplophase it is called "diplontic selection" (Gaul, 1957). The final result is a lower mutation frequency and a restricted mutation spectrum so that drastic chromosome aberrations and cells, which carry mutations for compact growth, dwarfness and the like, disappear automatically. When the breeding programme is aimed at gene mutations for certain characters without a reduction in growth, fertility and size, the restricted mutation spectrum is advantageous. When, however, the programme is aimed at the induction of chromosome breaks and the resulting aberrations or when chromosome engineering (e.g. the transfer of a piece of chromosome or gene from a wild species to the genome of a cultivar) is wanted, the "self-improvement" by this intrasomatic competition is undesirable (see also Section 1.3).

Moreover, since most apices consist of a number of fairly independent groups of cell layers, surviving and dividing mutated cells lead to the formation of mericlinal and periclinal chimeras. The chimeral situation is discontinued in seed propagated species after selfing or crossing; in vegetatively propagated plants, however, it is preserved as so-called budsports which are directly recognizable when mutations of visible characters are involved. Sometimes mutations, concerning characters of the outer layer(s), such as flower colour, cannot express themselves because they were induced in the inner layers. They may be detected years after the induction as the result of spontaneous or radio-induced uncovering or rearrangement, by which mutated cells appear in the outer layer(s). This phenomenon even has possibilities for practical application since the various combinations of mutated and non-mutated cell layers may result in different phenotypes (Bergann, 1966, 1967; Dommergues & Gillot, 1964; Klöpfer, 1966; Pötsch, 1966).

Both, diplontic selection and chimera formation very much restrict the potential of practical mutation breeding as well as more basic studies using induced mutations in plant material. Such an undesirable situation can to a great extent be avoided by growing complete plants from single cells, automatically resulting in a high(er) frequency of solid, non-chimeral mutants and a wide(r) mutation spectrum (see Section 1.4).

1.2 RADIATION TYPE AND METHOD OF IRRADIATION

Various types of ionizing radiations, such as electromagnetic radiation (X-rays, gamma-rays) and charged or uncharged particles (α-rays, β-rays, protons, neutrons) are used in radiobiological experiments to study the interaction between radiation and biological material (Bacq & Alexander, 1961).

In this study, *X-rays* were used as a representative of the sparsely ionizing electromagnetic radiations with a very short wavelength. The energy is almost entirely dissipated by electrons ejected from the atoms of the material through which they pass. This process is almost independent of the manner in which these atoms are combined into molecules and of its elementary composition.

The penetration of the X-rays depends on the energy used and should be high enough to prevent a "one-sided effect", as the result of an uneven dose distribution within the material. The X-rays, used in this study, had an effective energy of 50-60 keV, when the machine operated at 250 kVp and no additional filter was applied (the filtering of the apparatus was equivalent to 2-3 mm Al). The decrease in dose rate, caused by one, two or three layers of African Violet leaves, was measured, and turned out to be 0.9%, 2.5% and 3.5%, respectively. Since in all experiments a one-layer formation was used, where the leaves overlapped partly at most once, the low percentages indicate that the energy distribution within the leaves was sufficiently even.

Fast neutrons, having an energy of approx. 10 keV and up, were applied as a representative of more densely ionizing corpuscular radiations. Fast neutrons do not produce ionizations directly but mainly react with nuclei of the atoms of the absorbing material. The reaction with the H-nuclei is the most important, producing recoil protons which cause ionizations, concentrated along short tracks, inside the material. Therefore, unlike sparsely ionizing radiations (such as X-rays), the number of ionizations produced depends largely on the nature of the elementary composition of the material, hydrogen being the most important element when using fast neutrons (Bacq & Alexander, 1961; Dertinger & Jung, 1969). As hydrogen is the major constituent of biological materials, 85-95% of the energy is transferred by the recoil protons. In *Saintpaulia* leaves, with a water content of 97-98%, the hydrogen concentration, as well as its distribution throughout the leaves guarantees an even energy dissipation in the material (Dertinger & Jung, 1969).

Another reason to compare the effect of X-rays and fast neutrons is that sparsely ionizing radiation has a mainly indirect effect, whereas densely ion-

izing radiation has a mainly direct effect. The (intracellular) direct and indirect effects are of comparable magnitude in most cases, and depend on the cell function studied. The definition of direct and indirect varies and is rather confusing and to a certain extent meaningless. Some authors refer to the direct effect as an energy dissipation in the molecules involved, whereas in the indirect action these molecules are affected by radiation induced free radicals (see also Section 1.1). Hence X-rays have a stronger physiological effect and cause fewer chromosome aberrations than fast neutrons (Ehrenberg & Nybom, 1954; van Kampen, 1959; Stone, 1955). Other authors consider the unmodifiable damage as resulting from the direct action and the modifiable damage as resulting from the indirect action (Dertinger & Jung, 1969). Sometimes the direct effect is referred to as the effect upon two separate sites at the same time by the same electromagnetic ray or particle. This is more likely to happen with densely ionizing radiation, such as neutrons, than with sparsely ionizing radiation, such as deep therapy X-rays (Chadwick & Leenhouts, 1972). In the following part of this study it will be observed that the difference between the fast neutrons and the X-rays used, is much less than generally reported in the literature. Whether this is because most experiments were carried out with 14 MeV neutrons is not known, but seems improbable, since 14 MeV neutrons have a lower LET (linear energy transfer) and RBE (relative biological effectiveness) (Bacq & Alexander, 1961) compared with fast neutrons from the degraded fission spectrum, with an average energy of approx. 1.7 MeV, used in this study (see Section 2.1).

Various methods of irradiation have been used, namely acute, chronic or fractionated exposures with X-rays or fast neutrons.

Acute irradiations with X-rays were almost exclusively carried out with a dose rate of 200 rad/min so that radiation doses of 2-6 krad required only 10-30 minutes exposure times. The fast neutron dose rate (flux density) applied was equivalent to 1000 rad/h, so that exposure times were 2 to 6 hours. Since neutrons were not expected to have a dose rate effect, little attention was paid to this difference at first. But the experimental results as well as a few irradiations with higher dose rates showed that the dose rate of 1000 rad/h, used in most experiments, was at the borderline between acute and semi-chronic irradiations (see also Section 3.2).

Chronic irradiations with X-rays or fast neutrons have revealed a striking dose rate effect in *Saintpaulia* (Broertjes, 1968b). Dose rate phenomena, on which voluminous literature is available, have been investigated extensively,

either in mammalian systems (Anonymous, 1968) or in botanical material (Constantin et al., 1970; Sparrow, 1965). From the data presented it appears that low rates are less effective than high rates for a given total exposure. This holds true only within certain limits however: above a given dose rate a further increase does not result in an additional increase in effect. Similarly below a certain low dose rate a further decrease in rate does not decrease the effect (Bottino & Sparrow, 1970; Constantin et al., 1970; Sparrow et al., 1970).

Somewhere in the range between a low and a high dose rate, there is a critical area which represents a transition from a chronic irradiation (low dose rate) to an acute one (high dose rate).

Constantin et al. (1970) determined this so-called rate saturation for soybean seedlings and for mature plants as 50 R/min and 25 R/min, respectively. These results indicate that the phenomenon is dependent on stage of development during irradiation. The end-point measured and the total exposure also proved to be important factors.

Furthermore the rate saturation seems to be dependent on the radiosensitivity of the plant species involved: sensitive species would undergo a saturation at lower exposure rates than tolerant species. On the basis of their interphase chromosome volume, rate saturation constants for a number of species were calculated and compared with actual dose rate effect data, which seem to support the idea (see Section 3.2 for more details).

Dose fractionation was applied to collect information which could be used in explaining the striking dose rate effect mentioned before.

Dose fractionations, reported in the literature by many authors, are carried out in various ways. Sometimes a total dose is compared with two fractions half its size, separated by various time intervals. In other cases a certain dose is preceeded by various initial doses, separated by one or more (different) time intervals. And still in other cases the effect of a series of fractions is studied (Bacchetti, 1965; Bacchetti et al., 1965; Bacq & Alexander, 1961; Bewley et al., 1963; Bryant, 1968; Davies & Wall, 1960; Evans, 1966; Evans, 1967a; Fujii, 1968; Generalova, 1968; Generalova, 1969; Howard, 1968; Kabakova et al., 1970; Kada et al., 1966; Kiefer, 1966; Lawrence, 1968; Leonard & Deknudt, 1971; Morris & O'Grady, 1970; Paribok & Krupnova, 1970; Tates, 1968; Tazima & Onimaru, 1966; Withers & Elkind, 1969; Würgler & Matter, 1968).

Generally the biological effect of fractionation is less than when the total dose is applied. This could be explained by part of the damage of the first fraction being repaired before the following dose(s) is applied.

In *Saintpaulia*, using the adventitious bud technique (see Section 1.4), both X-rays and fast neutrons were used to study various elements in fractionation experiments: initial dose, time interval, second dose and repeated irradiations (see Section 3.3) (Broertjes, 1970).

1.3 DIPLONTIC SELECTION

The mutation frequency, expressed as detectable mutated sectors after irradiation of multicellular apices of African Violet plants, was shown to be extremely small in the *Saintpaulia ionantha* cultivar "Utrecht". During one of the first experiments 100 whole plants were irradiated with 3 krad X-rays (dose rate 300 rad/min). Only one mutated (flower colour) sector was observed a few months after regrowth and further development. This observation agrees with the relatively low number of spontaneous mutants in the greenhouses of *Saintpaulia* growers. Similarly *Achimenes* and *Streptocarpus*, two other representatives of the *Gesneriaceae*, have few spontaneous mutants. Although it is claimed that the hundreds and hundreds of cultivars of *Saintpaulia ionantha* are the result of both hybridizing and selection among mutants (Moore, 1957), recently no mutants have been commercialized in Western Europe. In *Streptocarpus* a white-flowering mutant, e.g. "Maassens White", arose spontaneously in the blue-flowering cv. Constant Nymph, whereas in *Achimenes* the only mutant recently commercialized was the cv. Willem Maarse, with bright green leaves and stems (the original form had darker green leaves).

In strong contrast to these facts are the high mutation rates among the adventitious buds developing at the base of the petioles of irradiated leaves (> 20% after an X-ray dose of 3 krad). This difference can be explained on the basis of diplontic selection, in combination with factors related to the structure of a multicellular apex (Dommergues, 1962; Gaul, 1963) and consists of the following elements:

1. the general competition between cells in a multicellular apex also as a consequence of its structure. The apex of angiosperms consists of three fairly independent cell layers, L_1, L_2 and L_3 (Satina et al., 1940), each containing a restricted number of initial cells and a number of less active cells (méristème d'attente) (Buvat, 1952). Extensive information and details are given by Balkema (1971), Dommergues (1962) and Yamazaki (1963). It is obvious that the destination of a mutated cell is defined by its role within the apex and that a mutation in a cell within a group of cells, not taking part in the formation of the shoot, plant or axillary bud, will never be observed.

2. This also applies to mutations induced in the wrong cell layer. A flower colour mutation induced in one of the initial cells of the L_3 will, under normal conditions, never manifest itself.
3. The diplontic selection, in a more restricted sense, is the competition among mutated cells or between a mutated cell and its surrounding non-mutated ones. It will result in the survival of the fittest since even small differences in division rate cause the elimination of the more slowly dividing cells (Gaul, 1957).

The idea of diplontic selection has been opposed by a number of authors and was recently reviewed by Balkema (1971). She claims that the destiny of most cells in a multicellular meristem is already fixed and that diplontic selection plays a role in such cases, where only a few of the many potential organs can develop, such as with side shoots. In most cases, in her opinion, diplontic selection can be explained as aspects of normal development or as the result of differential (mutagen) sensitivity. She demonstrated in *Arabidopsis* and sunflower, that by changing the environmental conditions, chimerism was influenced and so could be manipulated.

Diplontic selection in adventitious buds, however, would appear to be restricted exclusively to point 3, since neither point 1 nor 2 apply to buds originating from single cells. It therefore remains to be explained which factors are responsible for the striking difference between the extremely low mutation frequency after the irradiation of plants and the high percentage of mutants, observed after the irradiation of detached leaves and the subsequent development of adventitious plantlets at the base of the petioles.

An examination of the striking difference in mutation recovery reveals a few interesting facts. The high mutant frequency, among adventitious plantlets 20% or more after a medium dose, indicates that the majority, if not all, of the cells must carry one or more mutations.

The 20% mutants are exclusively based on visible changes. There is no reason to assume that the genes controlling not-directly detectable characters do not mutate. The fact that we do find "unmutated" plantlets only might mean that in these plants no detectable mutations are present. It consequently appears that competition, if any, does not only take place between non-mutated and mutated cells among the few thousand epidermal cells which are candidates for the formation of adventitious buds, but that it mainly takes place among mutated cells. Here, too, a number of cells will be eliminated or are damaged so badly that they will not be able to form buds.

This, however, does not explain the difference between adventitious buds and multicellular apices, since the same consideration holds true for the latter: there is no reason to assume that the number of mutations will be lower in such apices. Is it due to the fact that solid mutants, obtained when using the adventitious bud technique, are recognized more easily than sectors, observed after the irradiation of multicellular buds? This could indeed apply to a number of characters (small changes in size, form or habit) but certainly not for others (colour of leaf and flower and extreme changes in size, form and habit). Or is the difference due to a much higher selection pressure in the multicellular apex, together with the more strict organization within and between the various layers and the reduced number of initial cells?

Cytological-histological observations of leaf petioles have revealed that meristems are formed at random around the circumference near the base of the petiole (on the dorsal side of the petiole slightly less meristems develop). It therefore seems logical to assume that all epidermal cells in that region have, in principle, an equal chance to become the origin of an adventitious bud, except those which are too heavily damaged. Photograph 2, presenting part of a cross section through the base of the petiole approx. 10 days after cutting off, shows that most epidermal cells have entered the division stage and have divided into two or more meristematic cells. Some cells, however, become omnipotent earlier than others and are the first to enter the division stage. From that moment almost nothing can stop the cells from becoming the origin of the apex of an adventitious bud. The surrounding cells, which also are stimulated to divide, seem to be controlled or suppressed by the mitotic wave, induced by those first cells. They form the base of the meristem as well as the connecting tissue between the adventitious bud and the inner part of the petiole. Photographs 3, 4, 5, 6 and 7 show various developmental stages of the adventitious bud formation.

One can ask why certain cells become the origin of a bud and others do not. It is a fact that only a very restricted number of possible candidate epidermal cells are "chosen": there are approx. 300 epidermal cells around the circumference of an average petiole which makes 3000 candidates when the lowest 10 cells are stimulated. Since ultimately about 15 buds are formed, only 0.5% of the cells have a chance to express themselves.

The question arises whether a certain selection pressure is present during the very first days after cutting off or if epidermal cells really are "chosen" at random. It is hard to tell whether the places of origin are predestinated or just at random, since no evidence has been found that shows a

relationship between place of origin and position of other elements in the petiole, such as the vascular bundles for instance.

The results after the irradiation of leaves seem to indicate that the diplontic selection is very restricted. As appears from the figures in Table 7 (page 32) a large percentage of the mutants observed belong to the category of dwarf mutants. This demonstrates that even slow growing mutant cells have ample chances in the competition with faster growing (unmutated) cells, unless one assumes that almost all cells carry a (great) number of mutations or chromosome aberrations and that the number of "normal" cells is extremely small.

This train of thought however is counteracted by the fact that small dosages, perhaps small dosages in particular, result in a large percentage of dwarf mutants (Table 7). The low mutation frequency at such a dose, approx. 5% after 0.5 krad (see Chapter 3) indicates a sufficient supply of normal, unmutated and undamaged cells, which could have suppressed completely the formation of slow growing dwarf types, even in the case of a slight diplontic selection in favour of the normal cells.

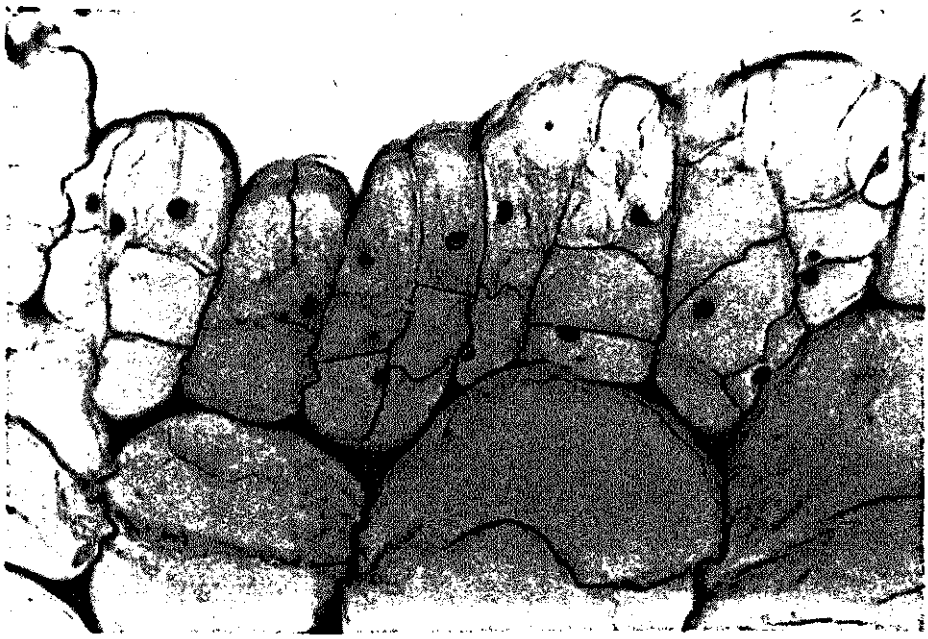
In a multicellular apex, however, the situation is completely different. The organization is much stronger and the number of initial cells much smaller. But the main difference is that the diplontic selection continues during the entire further development and growth of the shoot. Then even extremely small differences in division rate or general fitness ruthlessly eliminate and replace the weaker or slower growing cells. Such a high pressure during hundreds of cell divisions, in combination with points 1 and 2, leads to plantlets which resemble the original type in the majority of cases.

1.4 THE ADVENTITIOUS BUD TECHNIQUE

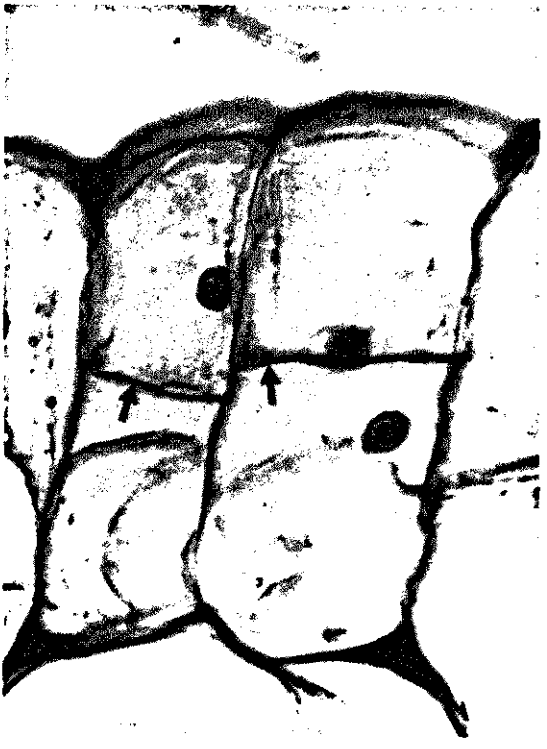
Mutation breeding offers large possibilities in vegetatively propagated plants, such as ornamentals, numerous fruit crops, potatoes, sweet potatoes, sugar cane, cassava and grapes. The main advantage is the possibility of improving one or only a few characters, of an otherwise excellent cultivar, without altering the remaining genotype. Thus the outstanding cultivars, often the result of a time-consuming and painstaking cross-breeding programme, can be further perfected within a reasonable time (Broertjes, 1968a; Nybom, 1961; Nybom & Koch, 1965).

Such cultivars are often very complicated genotypes which seggregate after selfing or hybridizing in a very complex way. This makes it, practically

Photograph 1. Leaves of *Saintpaulia* showing developmental stages of adventitious buds at the base of the petiole.

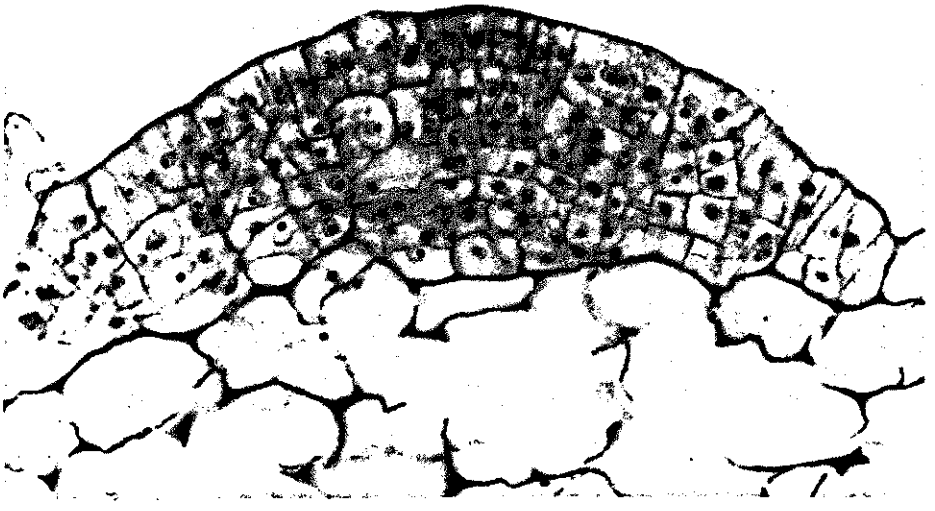


Photograph 2. Part of a cross section through the base of the petiole approx. 10 days after detaching. Most epidermal cells have divided.

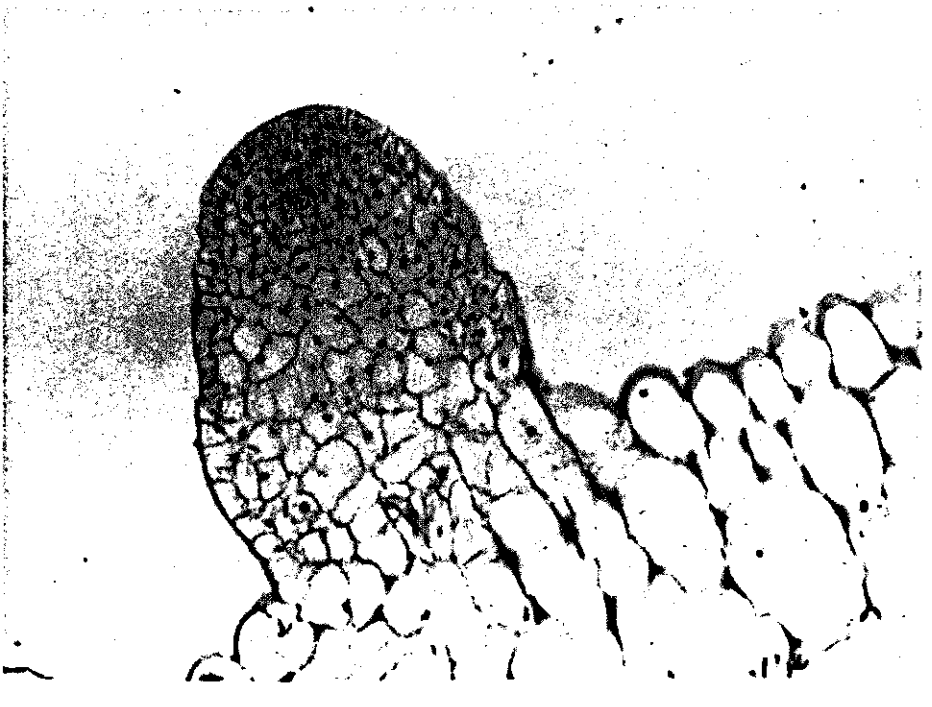


Photograph 3. First periclinal divisions (arrows) in epidermal cells at the base of the petiole of the leaf (approx. 5 days after the removal of the leaf).

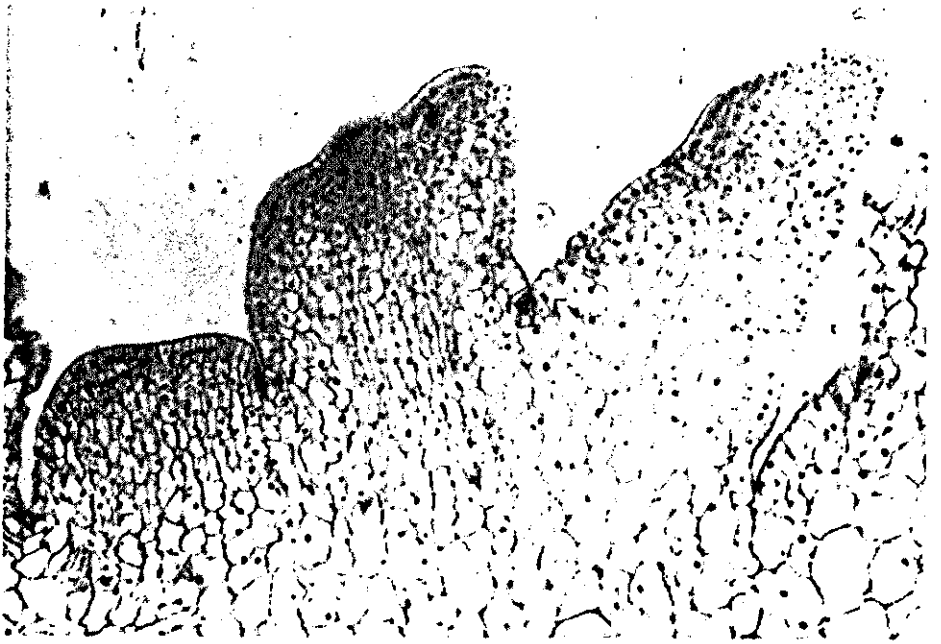
Photographs 4-7. Various later developmental stages leading to the formation of a meristem (after approx. 10 days; Photograph 5) which forms an apex which develops into a plantlet (Photographs 6 and 7).



Photograph 5.



Photograph 6.



Photograph 7.

Photograph 8. *Saintpaulia ionantha* cv. Utrecht, clone B.

speaking, impossible to recover genotypes in the offspring with the original gene combination, which, in addition, must moreover carry one or two favourable changes. Recovery becomes even more complicated and consequently improbable when polyploidy is involved. When only one or two (dominant) simplex characters have to be changed into a nulliplex, mutation breeding is an almost ideal tool. This in contrast to the situation where duplex, triplex or quadruplex (dominant) characters must be mutated. The irradiation of colchicine-induced autotetraploid plants, which are more radioresistant, yielded however surprisingly high percentages of mutants in *Achimenes* (Broertjes, 1972b), *Saintpaulia* and *Streptocarpus*. The higher radioresistance results from the fact that all kind of gross chromosome aberrations, lethal on diploid level, are not lethal in (auto)tetraploids. The high mutation frequency is attributed to the increased number of gross chromosome aberrations, which moreover have a dominant expression. In addition the chance is increased that identical genes in the two homologous chromosomes are involved in the mutation. Duplex genes (most are duplex, because of the heterozygosity of the original material) thus become nulliplex and manifest themselves in the case of visible characters.

The selection of visible characters generally offers no problems and a favourable change may soon lead to the commercialization of the mutant. This holds true for visible changes in ornamentals but also in other crops, such as fruit colour and spur type in apples and pears, skin colour of potato tubers as well as growth pattern, size, form and many other directly perceptible characters in general (Broertjes, 1971; Heslot, 1964).

It is therefore not surprising that the majority of the commercial mutation breeding projects, started since 1960 as co-operative programmes between the Association Euratom-ITAL at Wageningen and plant breeders or institutions, deal with vegetatively propagated plants, mostly ornamentals, but also fruit crops and potatoes (Broertjes, 1965; Broertjes, 1968a).

All kinds of plant parts are irradiated, for example bulbs, tubers, rhizomes, cuttings, grafts or (small) plants, all of which have in common that they carry buds with multicellular apices which generally are composed of a number of fairly autonomous cell layers.

As has already been discussed before, the irradiation of such a complex apex leads automatically to the formation of chimeras as well as to a low mutation frequency as the result of diplontic selection. This, the main stumbling-block, can only be overcome by growing plants from single cells, in vitro or in vivo, resulting in a higher frequency of solid, non-chimeral, mutants. It is therefore of utmost importance to look for and to develop such

methods.

For the commercial plant breeder the most important method at present is the adventitious bud technique, which makes use of the phenomenon that adventitious buds on detached leaves ultimately originate from only one (epidermal) cell (Broertjes et al., 1968) as has not only been shown in *Saintpaulia* (Naylor & Johnson, 1937; Sparrow et al., 1960; Broertjes, 1968b), but also in *Achimenes* (Broertjes, 1972b), *Kalanchoë* (Broertjes & Leffring, 1972), *Streptocarpus* (Broertjes, 1969) and tobacco (de Nettancourt et al., 1971). In all these cases it has been demonstrated, by mutation induction and cytological-histological observations, that neoformation takes place in several layers. In the L_3, meristems are formed which develop into root primordia whereas in the epidermal layer (the L_1) cells which have become dedifferentiated and meristematic, develop into shoot primordia after the formation of a meristem (photographs 4-7). Similar phenomena have been observed in other species, such as in *Lilium* hybrids, which almost exclusively produce solid mutants after the irradiation of bulb-scales, preferably removed from the bulbs after irradiation (Broertjes & Alkema, 1970). In *Endymion*, which like *Lilium* belongs to the monocotyledons, cytological-histological investigations have revealed no principle differences in the development of adventitious bulbils at the base of detached leaves, compared with adventitious plantlets in the dicotyledons mentioned before (Broertjes, in preparation).

Such adventitious buds are exclusively formed by cells at the lowest part of the petiole near the cut end (see Photograph 1). During all our experiments, as a routine, the basal 5 mm of the petiole was cut off after the completion of the treatment and prior to planting. In this way it was made sure that all epidermal cells which might have entered the division stage as a starting point for the formation of adventitious buds (approx. after 5 days) or meristems in more advanced developmental stages (following prolonged treatments) were removed. Consequently, chimera formation and cell competition or cell elimination (as a result of differences in radiosensitivity related to variation in metabolic activity, cell division stage or lethal chromosome rearrangement) was avoided during the treatment. In this way, higher situated epidermal cells on the petiole, which have not yet been stimulated to divide, were stimulated to do so by the removal of the 5 mm long basal part, after the irradiation had been completed. All such epidermal cells consequently have undergone the whole treatment in a resting, non-dividing stage of development.

The question arises in which stage of the nuclear cycle were these cells.

Was it G_1, the pre-DNA synthesis phase, the S-phase, during which DNA is synthesized, or G_2, the post-DNA synthetic period, prior to the actual mitosis? It could also have been a mixed population of cells in different phases of the cycle. Although labelling with radioactive thymidine and DNA cytophotometry were not applied to investigate the stage of the nuclear cycle, there are some considerations, which indicate that most, if not all epidermal cells, were in G_1. One is the fact that an overwhelming majority of the adventitious mutants observed in *Saintpaulia*, *Achimenes*, *Streptocarpus*, *Kalanchoë* and other species, were non-chimeral. Irradiation of cells in the (late) S-phase, when the cells are in G_1, as far as the DNA structure, but in G_2, as far as the amount of DNA is concerned, would have resulted in a much higher percentage chimeras. Furthermore it is increasingly frequently reported, that in some species all meristematic cells in an embryo of a seed cumulate in G_1 during seed maturation (*Pinus pinea*, *Lactuca sativa*, *Helianthus annuus*). In other species, however, cells were almost exclusively in G_1, or in G_1 and G_2 in different proportions (d'Amato, 1972).

If the epidermal cells and probably also other cells of the *Saintpaulia* leaf, indeed appear to be in G_1 (which is under investigation), it would represent an ideal synchronization-method, which could profitably be used in radiobiological experiments. Synchronization of animal or plant cells generally is achieved by careful handling of temperature fluctuations, to which the cell cultures are exposed, or by using certain chemicals, such as 5-aminouracil, which block DNA-synthesis without interfering with the preceeding phases. After thorough washing, all cells start DNA-synthesis simultaneously. Within a short period, however, desynchronization takes place (Clowes, 1965; Prensky & Smith, 1965; Sybenga, 1968).

It is obvious that only by painstaking and laborous procedures can a satisfactory synchronization be obtained and one wonders whether the use of leaves might be an easier and less expensive method.

It is concluded that the use of leaves of African Violet can be considered a clean method, to study radiation effects and to demonstrate its significance for applied mutagenesis. The most important reasons are the easy reproduction of the plantlets, ultimately originating from only one epidermal cell, the subsequent prevention of chimera formation and the decreased or almost eliminated consequences of differences in radiosensitivity as the result of different nuclear cycles of the (epidermal) cells.

Many plants can be propagated from adventitious plantlets on detached

leaves. Broertjes et al. (1968) list over 350 species, including a variety of families, reported in the literature, that belong to that group. Many, however, are not listed, including a number of economically very important plant families, such as *Gramineae* (cereals) and *Solanaceae* (potato, tomato). Many plants not listed have been tried without success. Many more have never been tried and the breeder should always experiment with both the species and the cultivar(s) he is interested in, since it has been shown that the adventitious plantlet production varies greatly among different cultivars of one species. In *Kalanchoë*, for instance, the cultivar Annette produces fewer plantlets than the cultivar Josine (Table 1) (Broertjes & Leffring, 1972). Similar differences have been reported by private breeders using various *Chrysanthemum* cultivars (pers. commun.). One should therefore not conclude that the technique does not work in a given species when only one variety or cultivar has been tested.

When testing the ability for adventitious bud formation, variables like rooting-medium (sand, peat, soil, vermiculite or mixtures), environmental conditions (temperature, humidity, light) and the use of hormones to promote rooting, should be included. One should not overlook various leaf factors, such as the age of the leaves which proved to be decisive in *Kalanchoë* (Broertjes & Leffring, 1972). In this study old leaves, just mature leaves and young leaves were compared. Although all leaves rooted readily, young leaves produced many more adventitious plantlets and produced them sooner, at the base of the petiole, than older leaves. The leaf petiole also seems to play a role: leaves of the *Kalanchoë* cultivar Annette rarely produced plantlets when the petiole was cut off, whereas the cv. Josine produced some plantlets on leaves of all ages, although many more on those with a petiole (see Table 1). (Leaves without a petiole root and develop plantlets almost exclusively near the midrib.)

Table 1. Number of adventitious plantlets on leaves of two *Kalanchoë* cultivars[1].

	cv. Annette		cv. Josine	
	with petiole	without petiole	with petiole	without petiole
Young leaves	27	-	73	24
Almost mature leaves	20	3	40	5
Old leaves	-	3	47	13

1. In all cases 20 leaves were used. All leaves were irradiated with 2 krad X-rays. The number of plantlets was determined 7 months after the start of the experiment (March 1970 - November 1970).

Table 2. Number of adventitious bulbils on leaves and leaf-parts of *Ornithogalum thyrsoïdes* Jacq.

	Number of leaves	Length (cm)	Number of bulbils per leaf or leaf-part
Whole length	10	20	16.2 ± 1.4
Base	20	10	6.7 ± 0.4 larger bulbils
Mid-piece	20	10	8.4 ± 0.6
Top	20	10	8.8 ± 0.5

The size of the leaf as well as the leaf part is an important factor for the number of adventitious buds formed in monocotyledons. In *Ornithogalum*, leaves of approximately 20 cm length were compared with leaf-tops, leaf-bases and mid-leaf pieces of approximately 10 cm length. In Table 2 it can be seen that whole leaves produced twice as many adventitious bulbils as any leaf-piece. Bases produced the lowest number, but the bulbils were larger (Broertjes, 1972a).

The number of adventitious plantlets is also influenced by the way the leaves are used: in *Streptocarpus* the greatest number is obtained when the two leaf-halves are planted, after taking out the midrib, as compared to planting the whole leaf without petiole (Broertjes, 1969).

Furthermore the use of modified leaves should not be overlooked. In *Lilium* hybrids, such as the Mid Century hybrid cultivars Enchantment and Tabasco, bulb scales are widely used as propagation material. After irradiation and mutation induction thus far exclusively solid, non-chimeral mutants have been obtained. The use of these scales or, in other species, of artificial scales (by cutting bulbs into pieces) may offer possibilities, especially since the detrimental effect of fungi can now be very effectively prevented by new fungicides, during storage, rooting and bulbil formation. Moreover the use of wounded bulbs, a normal procedure to propagate Hyacinth for instance, also looks promising (Broertjes & Alkema, 1970).

The use of chemicals is another approach. Rooting is often promoted by the application of plant hormones but the factors which control adventitious bud formation are still unknown. Cyto-kinins and other plant regulators certainly play a role, as has been demonstrated in *Begonia* (Heide, 1965), but for plants, not naturally forming adventitious plantlets, so far no practical application is in sight (Bhojwani & Johri, 1970; Menhenett, 1970; Nag & Johri, 1970; Plummer & Leopold, 1970).

A convincing example of the value of the adventitious bud technique for the possibilities of mutation breeding in vegetatively propagated ornamentals

has been demonstrated in *Streptocarpus*, using the cultivar Constant Nymph. Irradiated or colchicine treated leaf-halves produced a strikingly high percentage of gene and chromosome mutants or genome mutants respectively: over 30% of the adventitious plantlets produced proved to be (solid) mutants when the optimum X-ray dose was 3 krad or (non-cytochimeral) tetraploids when the treatment was for 6 hours with 0.1% colchicine. In total 857 mutants were obtained from which 5 were commercialized, within 3 years after the start of the experiment (Broertjes, 1969).

In *Achimenes* similar results were obtained. Though less mutable, leaf irradiation of the cultivar Paul Arnold produced a number of solid, non-chimeral mutants. Within slightly more than 3 years it resulted in the commercialization of three mutants, namely two earlier flowering ones ("Springtime", and "Early Arnold", both approx. 3 weeks earlier than "Paul Arnold") and one compact mutant ("Compact Arnold") (Broertjes, 1972b).

These results demonstrate that mutation breeding of vegetatively propagated (ornamental) plants can really be a "short cut", provided the adventitious bud technique is available.

The considerations in the foregoing sections have been supported by experimental results with *Saintpaulia ionantha* H. Wendl., applying acute, chronic or fractionated treatments with X-rays or fast neutrons. The objective was to investigate which treatment yields the highest number of useful mutants.

2 Materials and methods

2.1 IRRADIATION FACILITIES

X-rays were applied with a Philips 250/25 deep therapy apparatus, usually operating at 250 kVp and 15 mA, without an additional filter. The dose rate applied was always 200 rad/min. Except for the temperature, which was kept at ± 20°C during the irradiations, no other (climatic) conditions were controlled.

X-ray doses were determined with a Philips Universal Dosimeter connected to a hose-shaped intracavity ionization chamber (Anonymous, 1963). They were cross calibrated by using LiF thermoluminescent dosimeters (Puite et al., 1972). The dose determined is in rads.

The quality of the X-radiation depends a.o. on the voltage applied, on the use of additional filters and on the own filtering of the tube. The X-ray machine used has its own filtering value equivalent to 2-3 mm aluminium. This means that the continuous X-ray spectrum is chopped off partially, in a sense that the very low energetic rays are missing, whereas with increasing energy a decreasing part of it is absorbed. The effective energy of an unfiltered beam consequently has shifted to a higher value and for the machine used was approx. 50-60 keV, when operated at 250 kVp without additional filter. An X-ray spectrum of such quality guarantees an even dose distribution within biological material (see Section 1.2).

Fast neutron irradiations were carried out in the sub core irradiation room of the BARN (Biological Agricultural Reactor Netherlands).

Fast neutron doses were determined using acetylene equivalent and muscle tissue equivalent ionization chambers. The γ-contamination of the fast neutron beam was measured with a magnox-argon ionization chamber. The fast neutron spectrum is similar to a degraded fission spectrum and has an average energy of 1.7 MeV. The γ-contamination amounts to 80 rad/h (Chadwick & Oosterheert, 1969).

The fast neutron doses are given as rads in water using the fact that $D_n\ (H_2O) = 1.35\ D_n(CH)$ (Anonymous, 1967).

When operated at full power, a dose rate of 1000 rad/h in H_2O was

obtained at the irradiation position in the irradiation room of the BARN.

As *Saintpaulia* leaves contain 97-98% H_2O and because the contribution to the dose from H is about 100x the one from C, N and O (Anonymous, 1967 p. 75), the dose rate in water may be considered as the dose rate in the material. Moreover the N-content was very low (in % of the dry matter as well as in % of the fresh weight, 2.85% and 0.07% respectively).

All fast neutron doses stated in this paper are without γ contamination; they therefore must be increased with 8% of the dose, divided by the RBE factor involved.

2.2 PLANT MATERIAL

Saintpaulia, belonging to the *Gesneriaceae*, has been selected as an experimental plant for various reasons. It forms medium-sized plants which can be grown without difficulty under normal greenhouse conditions throughout the year. The species reproduces easily from leaf cuttings which, after rooting, produce 10-20 plantlets per leaf from adventitious buds formed at the base of the petiole. These adventitious plantlets can be separated from the mother leaf and transplanted into boxes or pots and grown to maturity.

This reproduction system, as has been discussed before, has been chosen to avoid the consequences arising from differences in radiosensitivity caused by different cell division stages as well as to prevent chimera formation resulting from mutation induction in multicellular meristems. In general 20 leaves per treatment were used.

Saintpaulia originates from tropical East Africa, where it was discovered in 1892 by Baron Walter von Saint Paul. Most present day cultivars are diploid (2n=28) and are descendents of crosses between species and cultivars of *S. ionantha* and *S. confusa*.

The cultivar, which was used for the experiments described, was *Saintpaulia ionantha* H. Wendl. cv. Utrecht: a compact growing species with crenate leaves and blue-violet single flowers. Since the variability of the commercially grown plants was rather large, as a result of accumulated spontaneous mutations, a number of clones, each one originating from one single leaf, were produced. After testing their homogeneity as well as their reactions to a series of acute X-ray doses, one of them was selected and propagated vegetatively. Any plant, deviating from the type, was removed (clone B: see Photograph 8).

The plants, used for the production of leaves, were grown in a greenhouse

with a day temperature of 22-25°C and a night temperature of approx. 19-20°C. During wintertime extra light was applied (Philips TL MF 140 W/33 RS, Double Flux; 80 watts per m^2 from 6 h 00 to 22 h 00). The plants were grown in clay pots of 10 cm diameter in a mixture of leaf-mold and fertilizer enriched peat. Extra fertilizer was applied every two weeks during active growth of the plants (Pokon: 16% N, 21% P_2O_5, 27% K_2O; 3 g/l).

The leaves were irradiated in sealed polyethylene bags (21 x 23 cm; 0.1 mm thick; 20 leaves per bag) and remained in these bags during the whole treatment in the dark (except during handling and irradiation) at room temperature (approx. 20-22°C). Several bags with untreated leaves remained with the treated ones under the same conditions.

The main purpose of storing the detached leaves in plastic bags is to prevent water loss by evaporation. Polyethylene of 0.1 mm thickness has a permeability for water of 140 $cm^3 dm^{-2}$ per day atm^{-1} under standard conditions (20°C and 760 mm mercury pressure). The permeability for oxygen and carbon dioxide is 20 and 90 cm^3, respectively (personal information of the manufacturer). These values, however, depend on many factors, such as the type of machine on which the polyethylene is made, the time of the year (the values vary, for reasons unknown, from sample to sample). But no effect has been established, which could be attributed to these differences in value. Moreover, the circumstances during the use play a role: the permeability, when hanging free in air is much larger than that of packed bags. During the experiments the bags with leaves were stored in perspex containers between the treatments and packed upon each other.

Consequently, the loss of water was reduced considerably: at a relative humidity inside the bags of 50%, for instance, a loss of 10^{-2} mg/h per bag was calculated, whereas 20 leaves, free in air, lose 200 mg/h as has been measured. The result was that the relative humidity remained high during the whole treatment and that often condensation was observed.

Under these conditions leaves stayed alive for at least 3-4 weeks without any unfavourable effect on rooting, survival and on production of adventitious plantlets. On the contrary, the production seemed to be slightly stimulated by the storage in plastic bags for reasons unknown (Table 3). However, the differences are far from significant. All experimental results have been expressed in their respective controls, which were stored and handled in exactly the same manner as the leaves undergoing the irradiations.

The effect of the storage on the mutation frequency turned out to be nil: hardly any aberrant types were detected in control plants.

Table 3. Effect of duration of storage in polyethylene bags on survival of leaves and production of plantlets.

Storage time (weeks)	Number of leaves	Survival (%)	Number of plantlets	Number of plantlets per leaf
Control	50	94	804	17.1 ± 0.7
1/2	50	100	890	17.8 ± 0.6
1	50	96	883	18.4 ± 0.7
2	50	96	881	18.35 ± 0.6
3	50	94	818	17.4 ± 0.9
4	50	92	819	17.8 ± 0.7

The irradiated leaves were planted in a mixture of peat and leaf-mold (3:1) in wooden boxes of 37 x 37 cm, 5 rows of 8 leaves each. Later, foam plastic containers, 30 x 40 cm, were used. After rooting, as soon as the first plantlets emerged through the peat (3-4 months after planting), the surviving leaves were transplanted to similar sized boxes or containers. This time they were planted in 4 rows of 5 leaves each, in a mixture of leaf-mold and peat (3:1).

As soon as the plantlets were large enough, 3-5 cm tall, they were separated from the mother leaf, counted (per leaf) and planted in wooden or foam plastic boxes of 37 x 47 cm; each box containing 30 plantlets. After approx. 3 months the mutation frequency was determined by counting the number of visible mutants. The total time, needed per experiment, amounted to approximately 9 months, depending on the season.

Moreover the mutation spectrum was determined by classifying the mutants into a number of groups, such as:

Plant habit

large (generally the result of larger leaves)

rosette (shorter leaf-petioles combined with smaller leaves)

compact (short petioles and small leaves)

semi-dwarf (size of the plant approx. 50% of the control)

dwarf (size approx. 25%)

super dwarf (extremely small plantlets)

Leaf characters

long petiole and entire leaf (these two characters almost always appeared together)

colour (dark green, pale green, yellow, white, brown, speckled, spotted, variegated)

form (pointed, round, serrate, toothed or entire, uneven blade surface (corrugated))

size (ranging from very small to somewhat larger)
Flower characters
colour (different shades of blue and purple)
size (generally smaller to small; seldom larger)
form (from open to half-open to closed)
number (from free flowering to not flowering).

It was not always easy to classify a certain mutant, since there are no sharp and well defined differences between one group or another. Many of the mutants, carrying more than one mutated character, belonged in different groups.

2.3 PARAMETERS

Three parameters were used to measure the effect of the irradiation, namely:

1. The *survival* of the irradiated leaves (in % of the control). The majority of the leaves which were able to form roots, also produced one or more adventitious plantlets. Sometimes, however, after a heavy treatment, a leaf only formed one little root and therefore stayed alive. However, such a leaf will never produce a plantlet and moreover will die sooner or later. It therefore was considered to be dead and only those leaves which produced at least one plantlet were considered to be alive. Since a number of experiments were not repeated, or repeated only once, the statistical variance of survival was not determined.

However, from all experiments, a series of observations were obtained (e.g. survival and a series of increasing doses or, survival and a series of increasing time intervals, etc.). Although the individual significance of a given observation cannot be indicated, it can be estimated from its position with respect to the neighbouring observations. The distance to the general line therefore is a measure of its significance and could be expressed as an amplitude or as a mean deviation. Both are rather meaningless and this method of expressing significance has been abandoned.

A general disadvantage of this parameter is that it does not reflect differences in reaction among treatments which resulted in 100% survival, in contrast to the next parameter (production), which sometimes showed a stimulation effect.

2. The number of plantlets produced by each surviving leaf was counted. These figures were then used to calculate the mean and the variance. This is termed

Table 4. The relation between the average number of plantlets per leaf (production) and time of year when leaves are detached.

Time of detaching the leaves	Number of leaves	Av. number of plantlets per leaf
February	60	16.9 ± 0.5
April	60	13.4 ± 0.4
July	60	11.1 ± 0.5
October	60	9.7 ± 0.4

production and is expressed as the average number per surviving leaf as a % of the control. This is the most reliable parameter and generally reacts very sharply to the intensity of the treatment. Untreated leaves produce approximately 15 plantlets per leaf, depending on the time of the year.

In a given set of experiments the average number of plantlets per leaf were as in Table 4. In some cases, however, and for reasons unknown, approx. 20 plantlets per leaf were produced.

In order to eliminate the effect of varying factors as much as possible, leaves of approximately the same age and size were used, whereas growing and treatment of the plants were standardized as much as possible.

Since survival and production were expressed in % of the control, a great part of the influence of time of year and other varying factors was eliminated.

Survival and production were determined 5-6 months after planting at the time of the separation of the plantlets from the mother leaf.

3. The *mutants*, directly visible aberrant types, were selected and counted approximately 3 months after the separation when the plantlets were large enough to distinguish variations in size, form, and colour of leaf, flower and plant habit. The number of mutants can be expressed in several ways, namely as the number of mutants per 100 plantlets, hereafter referred to as the *mutation frequency*, and as the *number of mutants per 100 irradiated leaves* or in other ways (see Section 3.1.1, and tables 5 and 6, page 28 and 29 resp.).

These parameters are the least reliable, since habit, size and other visible characters vary with differences in climatic and other conditions and, in addition, are influenced by the previous treatments. The decision of whether or not a plant was a mutant was occasionally an arbitrary one.

To investigate whether an aberrant type was or was not a mutant, a number of normal looking plants as well as mutants were propagated vegetatively, using the same technique as described before. All aberrant types proved to be stabilized phenotypes after clonal propagation and consequently were the result of genetic changes rather than temporary physiological disturbances. Some of the normal looking plants, however, could be distinguished from the

control when they were compared on a clone basis. Thus small changes in habit, size or otherwise must have been overlooked at first when comparisons were made on a one plant basis. Here, too, statistical analysis was not carried out for the same reasons as have been mentioned for survival.

Most of the data are presented using two linear scales in contrast to the log-survival curves generally used by other authors (Casarett, 1968; Neary, 1969). They worked with micro-organisms (bacteria, yeast), tissue cultures, or cell-suspensions of which even extremely low survival rates could be measured. Consequently the extent of the shoulder and the ultimate slope of the approximately linear later portion of the curves can be determined, which then are used for measuring extrapolation numbers as a basis for further calculations.

Leaves of a plant, *Saintpaulia* in this case, are not just a number of cells, more or less independent of each other, but are organs. When the percentage of cells, carrying too heavy or lethal damage, is higher than a certain (unknown) threshold, the whole leaf dies. For complete plants the threshold, and consequently the extent of the damage, is apparently higher than for detached leaves. Plants can easily stand doses up to 15 krad X-rays, as has been observed. They stay alive for a long period, although growth (neoformation) is suppressed completely. They become abnormally dark green, getting large and thick leaves and die, ultimately.

When detached leaves are irradiated the situation is different. Cells involved in root formation, which consequently have to divide, are much more radiosensitive: a much less severe radiation damage completely suppresses cell division and the subsequent neoformation. Then the whole leaf dies. (Survival is nothing else but shorthand for pertaining the ability to divide). Most experiments had to be restricted to 20 or at most 40 leaves per treatment, because of lack of greenhouse space. This is in strong contrast to the astronomic numbers which bacteriologists for instance can handle. Consequently, in my experiments where survival and production decreased rapidly between 4 and 6 krad X-rays, very insignificant or no data were obtained between 0 and 10%. Therefore log-survival-curves were useless since the slope of the linear portion of the curve could not be determined accurately enough.

3 Irradiation experiments

3.1 ACUTE IRRADIATIONS

As has been discussed in Section 1.2, X-rays and fast neutrons were used to study the interaction between biological material and the method of irradiation.

Acute irradiations with X-rays have almost exclusively been carried out with a dose rate of 200 rad/min. This dose rate is, as will be explained in Section 3.2, far beyond the rate saturation (see Section 1.2), or the critical dose rate (see Section 3.2) which both lie in a transition area (called semi-acute) between chronic irradiation (with low dose rates) and acute irradiation (with high dose rates). This critical area is, as we will see, dependent on the radiosensitivity of the material and consequently different for different species (Section 3.2).

Acute irradiations with fast neutrons were usually carried out with a dose rate of 1000 rad/h (approx. 17 rad/min). This rate, as will be demonstrated, is just about at the borderline between semi-chronic and acute (see Section 3.2). Since the RBE value (relative biological effectiveness) under these circumstances, using the parameters discussed before, is approx. 2, this dose rate can be compared with 34 rad/min X-rays, which is just about in the acute part of the curve (see Fig. 4). (The RBE value is used in radiobiology to assess the effectiveness of one kind of radiation relative to some "reference" radiation, usually 200 kVp X-rays. The value is defined as the ratio of the doses of the radiations producing the same biological response under identical conditions. It is not clear why RBE values for the same spectrum of neutrons vary so widely between species and for different biological end-points. The differences depend on dose and criteria used, such as survival, growth, cytogenetic and genetic effects (IAEA Panel, Vienna, Dec. 1971)).

3.1.1 *Physiological and genetic effects*

Fig. 1 represents the X-ray dose effect curves for the three parameters used, namely survival, production and mutation frequency, with a dose rate of approx. 200 rad/min. The curves show the normal pattern: there is a linear response for mutation frequency, as well as for survival between 4.8 and 6 krad and for production between 3-6 krad. The doses 3 krad (50% of the sub-lethal dose with 100% survival and approx. 90% production) and 6 krad (sub-lethal dose with approx. 20% survival and 10% production) have often been applied after one or more initial doses during fractionation experiments (see Section 3.3).

Fig. 2 represents the dose effect curves after fast neutron irradiations in the BARN; the dose rate applied was 1000 rad/h fast neutrons (and a γ-contamination of 80 rad/h).

Surprisingly enough the curves were almost identical with those when X-rays were used; this phenomenon was not only restricted to acute irradiation and will therefore be discussed in detail in Chapter 5. From figs. 1 and 2 it appears that the RBE value for the fast neutrons is approx. 2, a value which seems to agree reasonably well with those given in the literature (Bacq & Alexander, 1961 p. 92; Broerse, 1966 p. 93). Its value also indicates that the fast neutron dose rate was just about between semi-acute and acute when it is compared with the RBE(=3) value observed after the use of a higher dose rate (57 rad/min), being well in the acute part of the dose rate response

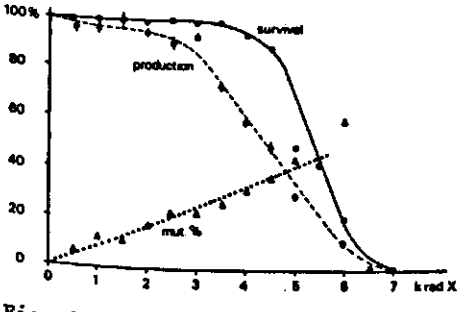


Fig. 1. Dose effect curves after acute X-ray treatments (200 rad/min) of African Violet leaves. Parameters: number of surviving leaves, as a percentage of control (survival), average number of adventitious plantlets per leaf, as a percentage of control (production) and number of mutants per 100 adventitious plantlets (mut.%).

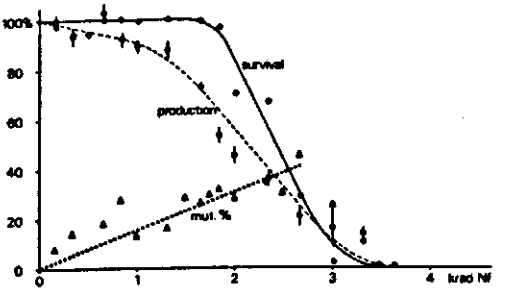


Fig. 2. Dose effect curves after acute fast neutron treatments (1000 rad/h) of African Violet leaves. Parameters: number of surviving leaves, as a percentage of control (survival), average number of adventitious plantlets per leaf, as a percentage of control (production) and number of mutants per 100 adventitious plantlets (mut.%).

curve (see Section 3.2.1 and Fig. 9).

The variance in figs. 1 and 2 for production is rather small, because the figures are based upon data obtained during many irradiation experiments. Every experiment, either acute, chronic or fractionated, always included a few standard acute doses, usually 3 and 6 krad X-rays or 1700 and 3400 rad fast neutrons. (At that time reactor dosimetry was in progress and the fast neutron doses mentioned, as well as the somewhat unusual doses used in fractionation experiments, were based upon preliminary dosimetry data, 1700 and 3400 rad being 100 and 200 min irradiation.)

These fairly reasonable standard deviations are in contrast with the much greater variance of the rest of the data obtained, which are based upon one or at most two repetitions of a given experiment.

From the figures it would appear that there was a slight stimulation in production at a low dose, namely at approx. 1.5 krad X-rays and 0.75 krad fast neutrons. Although it is convincing that both X-rays and fast neutrons showed this stimulation at comparable doses, calculations have revealed that the

Table 5. Values of various parameters after acute X-ray treatments.

Dose (krad)	Survival (%)	Production (% of control)	Number of plantlets per 100 irradiated leaves[1]	Number of mutants per 100 plantlets	Number of mutants per 100 irradiated leaves	Number of leaves to irradiate to produce 330 mutants
	A	B	$C=\frac{AxB}{100}x15$	D	$E=Cx\frac{D}{100}$	$F=\frac{330}{E}x100$
0	100	100	1500	0	-	
0.5	100	99	1485	4	59	559
1	100	98	1470	8.5	125	264
1.5	100	97	1455	12	175	189
2	100	96	1440	17	245	135
2.5	100	92	1380	21	290	114
3	*99*	*89*	*1322*	*25*	*330*	*100*
3.5	98	76	1117	29	324	102
4	96	62	893	33.5	299	110
4.5	87.5	48	630	37.5	236	140
5	64	35	336	42	141	234
5.5	38	20	114	46	52	635
6	10	8	12	50	6.0	5500
6.5	1	2	0.3	54	0.16	206250
7	-	-	-	-	-	-

1. On the basis of 15 plantlets per leaf of the control.
The data in column A, B and D, as well as the calculated figures in C, E and F, are the averages of all acute irradiations carried out (see text).
Figures have been rounded off to nearest whole number when below 1000 and to nearest 5 when above 1000.

difference between this point and neighbouring ones was not significant.

Then the question arises how to express the genetic parameter and how to manipulate the data on which Fig. 1 and Fig. 2 are based, to arrive at conclusive figures.

There are various ways to express the number of mutants observed:
1. number of mutants per 100 adventitious plantlets (mutation frequency; column D in tables 5 and 6);
2. number of mutants per 100 irradiated leaves (column E in tables 5 and 6);
3. number of leaves to produce a given number of mutants. This, the number of mutants, could have been 1000 but for X-rays 330 was chosen, which is the number produced by the optimum dose of 3 krad (column F); in the case of fast neutrons the optimum dose of 1.5 krad produced 269 mutants (both numbers apply for a given set of experiments).

It is evident that the highest possible mutation frequencies are not to be preferred. The enormous number of leaves needed for the production of for instance 1000 mutants (> 600.000!) is just not practical. Doses very near the

Table 6. Values of various parameters after acute fast neutron treatments.

Dose (krad)	Survival (%)	Production (% of control)	Number of plantlets per 100 irradiated leaves[1]	Number of mutants per 100 plantlets	Number of mutants per 100 irradiated leaves	Number of leaves to irradiate to produce 269 mutants
	A	B	$C=\frac{AxB}{100}x15$	D	$E=Cx\frac{D}{100}$	$F=\frac{269}{E}x100$
0	100	100	1500	-	-	
0.25	100	98	1470	4	59	456
0.5	100	96	1440	8	115	234
0.75	100	93	1395	11	153	176
1	100	91	1365	15	205	131
1.25	100	86	1290	19	245	110
1.5	*100*	*78*	*1170*	*23*	*269*	*100*
1.75	98	67	985	27	266	101
2	83	55	685	31	212	127
2.25	63	44	416	34	141	191
2.5	44	33	218	38	83	324
2.75	23	20	69	42	29	928
3	9	10	14	46	6	4480
3.25	3	5	2	50	1	26900
3.5	-	-	-	-	-	

1. On the basis of 15 plantlets per leaf of the control.
The data in column A, B and D, as well as the calculated figures in C, E and F, are the averages of all acute irradiations carried out (see text).
Figures have been rounded off to nearest whole number when below 1000 and to nearest 5 when above 1000.

lethal dose should be avoided although they show very high, but insignificant, mutation frequencies. (The number of adventitious plantlets, after very high doses, is extremely small which results in very unreliable figures for mutation frequency. They, therefore, have been omitted in further discussions). They also show an increased physiological effect, as is manifested by the slower rooting of the leaves and the delayed production as well as weaker growth of the plantlets. Unforeseen unfavourable (climate) conditions therefore could have a disastrous effect on the project after the application of such a heavy dose.

3.7.2 Practical implications

These considerations have led to the conclusion that the optimum treatment lies around the dose which results in a maximum number of mutants per given number of irradiated leaves.

In the case of X-rays this is 3 krad, producing 330 mutants per 100 leaves. In the case of fast neutrons it is 1.5 krad, producing 269 mutants per 100 leaves (see Fig. 3). It should be born in mind that the data used in the graph and the calculations in Table 5 and Table 6 are the averages of all experiments in which acute irradiations were applied; the absolute numbers differ from experiment to experiment as the result of differences in environmental conditions (time of the year and climate in general). During wintertime the effect of the treatment is somewhat stronger than in a more favourable season, resulting in a lower number of plantlets and a lower number of mutants per 100 leaves. This difficulty is overcome by expressing the results of any treatment in its corresponding 3 krad acute X-ray treatment.

A somewhat higher dose than the optimum dose of 3 krad could be chosen when the number of leaves available for irradiation is not restricted, but where the greenhouse space is the limiting factor. Then a higher concentration

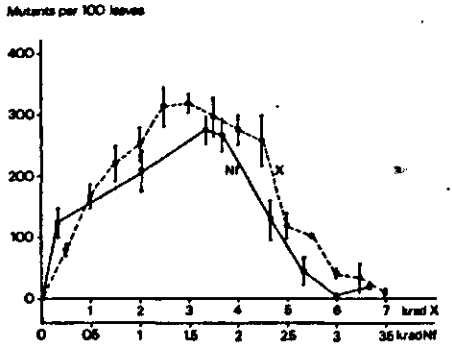


Fig. 3. Number of mutants per 100 leaves after acute X-ray (200 rad/min) and fast neutron (17 rad/min) treatments.

of mutants among the plantlets might be preferred, again keeping in mind the unpractical and limiting high number of leaves to be irradiated using very high doses. The optimum dose in such a case therefore lies around 4.5 krad X-rays or 2 krad fast neutrons.

Another conclusion which can be drawn from tables 5 and 6 is that fast neutrons apparently are less effective in mutant recovery, when the number of mutants per 100 leaves is used as parameter: 3 krad X-rays produce an average of 330 mutants per 100 leaves, whereas 1.5 krad fast neutrons produce only 269. However, all fast neutron experiments were carried out under normal conditions in the reactor, e.g. at 100 kW power, normal table height, normal climate conditions. Under these conditions a dose rate of 1000 rad/h (17 rad/min) was obtained, which, in comparison with X-rays, was low. Higher dose rates were applied during dose rate experiments, namely 3400 rad/h (57 rad/min) and 7750 rad/h (129 rad/min). The results of these experiments showed a somewhat different picture, namely an approx. identical number of mutants per 100 leaves (335 compared to 330 with X-rays), with a dose rate of 57 rad/min and a total dose of 1 krad. The RBE consequently was higher in such a case, namely 3 (see 3.2.1 and Fig. 9).

On the basis of number of mutants per krad fast neutrons are superior by far, at either dose rate.

During the experiments the impression arose that a heavier treatment caused a shift of the mutation spectrum towards unfavourable drastic mutants, as expressed by the % of compact, semi-dwarf, dwarf and super dwarf mutants (supposedly representatives of more drastic chromosome aberrations).

This idea, however, was disproved by testing the spectra of acute X-ray and fast neutron doses, with the total percentage of such plants taken from a random sample as a measure for the unfavourable part of the spectrum. These dwarf types are probably as phenotypes the summation of a combination of genetic disturbances and are moreover an indisputably, fairly large part of the spectrum.

As can be seen in Table 7 the average percentage is approx. 58-59% for X-rays as well as fast neutrons. There seems to be a tendency towards an even lower percentage at higher doses, which may be due to the elimination of part of the dwarf mutants (at the higher doses the totality of damage affecting growth would be greatest). Only extremely high doses, close to a lethal dose, resulted in 100% dwarfs. Since at these doses very few, very abnormal buds were formed, this figure was not significant and has therefore been ignored.

Table 7. Percentage of dwarf mutants after various doses of X-rays or fast neutrons.

X-rays			Fast neutrons		
dose (krad)	number of mutants (random sample)	dwarfs (%)	dose (rad)	number of mutants (random sample)	dwarfs (%)
Control	0	0	Control	0	0
0.5	41	73	170	158	64
1	195	66	340	104	77
1.5	51	63	500	45	60
2	281	59	670	31	61
2.5	316	67	840	115	59
3	1030	55	1000	108	57
3.5	576	55	1500	64	61
4	1013	54	1700	324	63
5	457	51	1840	118	36
6	62	44	2000	107	37
			2500	70	64
Total		587	Total		639
Average		58.7	Average		58.1

The deviating low percentages of dwarf mutants, after 1840 and 2000 rad fast neutrons, cannot be explained.

The overall high percentage of dwarf mutants indicates that the majority of mutants may be the result of drastic chromosome aberrations, rather than single gene mutations. In fact only a very limited number of mutants were found, among tens of thousands observed, in which only one visible character was changed.

3.2 CHRONIC IRRADIATIONS; DOSE RATE PHENOMENA

3.2.1 Physiological and genetic effects

With X-rays, chronic, semi-acute and acute irradiation treatments were compared, with dose rates ranging from 2 rad/min to 1000 rad/min. Still higher dose rates, e.g. 10 krad/min, were applied with electrons from a 1.5 MeV Van de Graaff electron generator. As can be seen in figs. 4 (survival), 5 (production) and 6 (mutation frequency) dose rates of approx. 20 rad/min and up, result in comparable effects for all three parameters. A drastic change seems to occur around 7 rad/min, separating acute and semi-acute effects (right part) from chronic effects (left part). This dose rate, 7 rad/min, has been described before as the critical dose rate (Broertjes, 1968b) and is comparable with what Constantin calls rate saturation (Constantin et al., 1970), although our data

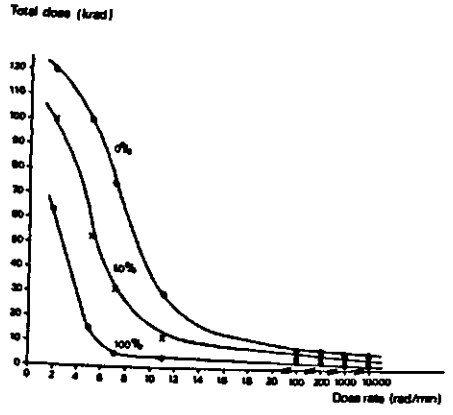


Fig. 4. Survival of irradiated leaves, as a percentage of control, after various X-ray dose rates.

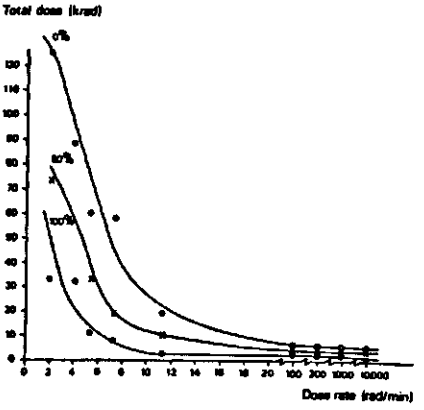


Fig. 5. Production of plantlets per leaf, as a percentage of control, after various X-ray dose rates.

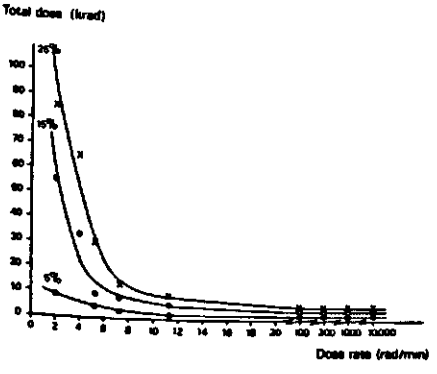


Fig. 6. Mutation frequency after various X-ray dose rates.

do not fit with his hypothesis (see Section 1.2). On the basis of interphase chromosome volume (ICV) of soybean and *Saintpaulia*, respectively being 2.95 mu^3 (Constantin et al., 1970) and 3.6 mu^3 (Sparrow, 1964) one would expect *Saintpaulia* to be more radiosensitive and to have a somewhat lower rate saturation than soybean. Both are out of line: *Saintpaulia* was less radiosensitive and the critical dose rate was much less than expected.

The disagreement between the ICV found in *Saintpaulia* and what would be predicted from the Constantin and Sparrow data may be explained in a variety of ways. The correct explanation requires considerable additional work. One possibility is that their conclusions do not have general applicability because of other complicating factors. Another is the variability of nuclear volume and interphase chromosome volume among cultivars of the same species and even within a cultivar at different times (Sparrow et al., 1961; Swift, 1953; Taylor, 1965; Yamakawa & Sparrow, 1965). Different ways of determining the interphase chromosome volume may play a role as well. We found an ICV of

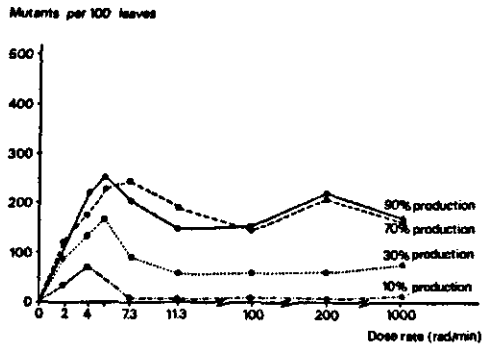


Fig. 7. Number of mutants per 100 leaves after various X-ray dose rates. (From Fig. 5 the doses can be read or interpolated, for any given dose rate, which had to be applied to reach the various production levels.)

3.1 mu^3 for *Saintpaulia*, using Craf I + II as fixative, by embedding in parafin and by staining with safranin and fast green. Sparrow (1964) used Craf III, Bioloid and methyl violet and orange G, respectively.

The critical dose rate is a fairly sharp breakpoint, in either of the three parameters used. Although the genetic effect similarly relates to the dose rate as does the physiological effect it opens up a possibility for the calculation of an optimum dose rate.

This is shown in Fig. 7, in which the relation between number of mutants per 100 leaves and the dose rate is presented, either when using a heavy total dose (10 and 30% production), a medium dose (50 and 70% production) or a light dose (90% production). (These doses can be read or interpolated for every dose rate from Fig. 5.) The graphs of Fig. 7 show two maxima, one at 5.3 rad/min and a total dose of 10.9 krad and a second, somewhat less pronounced one, around 200 rad/min. This second maximum is caused by a steady survival and production with increasing dose rate, but with a continuous rise in mutation frequency per krad (see Fig. 5 in Broertjes, 1968b) with increasing dose rate, reaching a maximum at 200 rad/min.

The first maximum, 275 mutants per 100 leaves, exceeds by 25% the second maximum producing 220 mutants per 100 leaves, the latter being the corresponding optimum acute X-ray treatment of 3 krad of the experiments involved.

With fast neutrons similar experiments have been carried out. In contrast to the literature (see f.i. Elkind, 1970) a clear dose rate effect was observed, although much less pronounced than that of X-rays. The breakpoint (critical dose rate; rate saturation) occurred between 4 and 8 rad/min, using survival as end-point, and around 8 rad/min with production and mutation frequency as parameters (see figs. 8, 9 and 10 in Broertjes, 1968b), which is in agreement with the X-ray data. Calculations showed a maximum number of mutants per 100 leaves at 8 rad/min, namely 317 at a total dose of 1.2 krad, which is 141% of the 225 mutants, produced by the corresponding optimum acute dose of 1.5

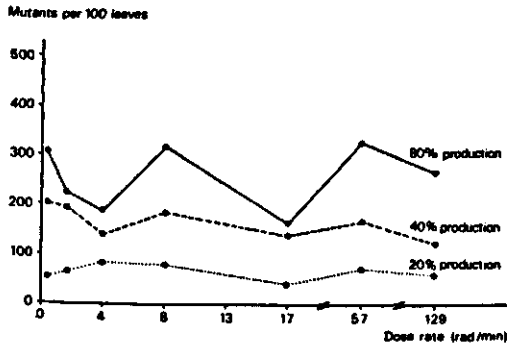


Fig. 8. Number of mutants per 100 leaves after various fast neutron dose rates.

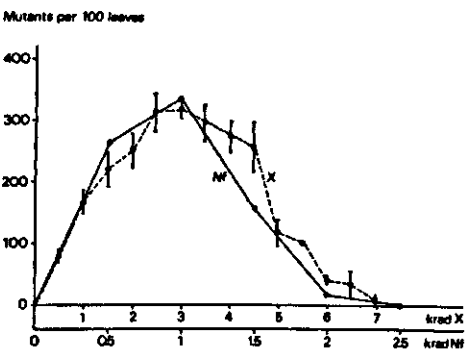


Fig. 9. Number of mutants per 100 leaves after acute X-ray (200 rad/min) and fast neutron (57 rad/min) treatments.

krad of the experiment(s) involved. Partly in agreement with the X-ray data is a second peak at a high(er) dose rate: 57 rad/min produced 335 mutants per 100 leaves after a total dose of 1 krad; this is 149% of the corresponding 1.5 krad. At the highest dose rate applied, 129 rad/min, the figures were lower. In disagreement is a third peak at the very low dose rate of 0.17 rad/min, namely 333 mutants after a total dose of 6.4 krad; this is 148% in comparison with the corresponding optimum 1.5 krad (Fig. 8). As has been said before, the fast neutron data were less pronounced than the X-ray results, which is also reflected by the 40% and 20% production lines in Fig. 8 that do not show any significant effect of the dose rates. The data therefore must be handled with care. The data also suggested that a dose rate of 57 rad/min could be better compared with the 200 rad/min X-ray treatment than the 17 rad/min used in most experiments. Also the number of mutants per 100 leaves of both dose rates are more comparable, as is shown in Fig. 9. From this figure it appears that neutrons and X-rays have a similar biological effect, that on a rad basis neutrons are much more effective and that in this case the RBE is approx. 3 instead of 2, found when using a dose rate of 17 rad/min (see Fig. 3).

The significance of the Nf-data in Fig. 9 is somewhat questionable because the fast neutron irradiation experiments with higher dose rates were carried out only once. (In the case of acute irradiations the final data are based upon a greater number of observations, such as in figs. 1 and 2, for instance.)

3.2.2 Practical implications

For the practical application of the data a number of considerations have to be taken into account. Not only must the treatment be efficient and result in a high number of mutants per 100 leaves, but it must be practical as well, which means that the type of treatment should be available.

With *X-rays* it is clear that low dose rates, 5.3 and 7.3 rad/min, result in a somewhat higher number of mutants per 100 leaves, namely 275 and 255, respectively, which is 125% and 116% of their corresponding optimum acute dose (3 krad, giving 220 mutants per 100 leaves; see Table 9, page 57). The spectrum at that dose rate seems to be unchanged: 58 and 53% dwarfs at 5.3 and 7.3 rad/min respectively, as compared to 55% at the optimum acute dose of 3 krad (Table 7, page 32). The duration of the treatment, approx. 20 hours at 5.3 rad/min (total dose 10.9 krad) and 18 hours at 7.3 rad/min (total dose 13.2 krad) is somewhat inconvenient but not impossible. A low dose rate can easily be made available with any X-ray machine. The conclusion therefore is that the optimum treatment is applicable for the plant breeder. Whether one should bother about the fairly small difference between this treatment and an acute irradiation is a different question, which may become relevant if irradiation costs play a role. If they are on a per hour basis, acute irradiation could be preferable.

With *fast neutrons* the situation is different. Provided a reactor, producing fast neutrons without a substantial γ-contamination, is available, it seems profitable to investigate the effect of different dose rates. Our figures suggest an almost 50% higher number of mutants per 100 leaves at a high dose rate (57 rad/min) as well as at a very low dose rate (0.17 rad/min), namely 335 and 333, which is 149% and 148%, respectively, of their corresponding optimal acute dose (1.5 krad) (see also Table 9, page 57). However, the mutation spectrum, which was not affected by dose rate when using X-rays, is influenced very much by the fast neutron dose rate, as appears from the figures in Table 8.

3.3 FRACTIONATED IRRADIATIONS

3.3.1 Physiological effects

The first experiment in which two equal semi-lethal doses of 3 krad X-rays each (50% of the sub-lethal dose of 6 krad X-rays) were applied, showed

Table 8. Percentage of dwarf mutants after various fast neutron dose rates[1].

Dose rate (rad/min)	Number of mutants	Number of dwarfs	Dwarfs (%)
0.17	243	110	45
1.7	398	215	54
4	86	40	47
8	207	112	54
17	1366	785	58
57	114	92	81
129	66	50	76

1. This table does not present data on the basis of mutants per 100 leaves but is merely a sample of the mutations taken from some experiments.

that after an interval between doses of more than 8-12 hours the effect of the two fractions was identical with the effect of only one fraction on both survival (Fig. 10) and production (Fig. 11). (The two data at 18 and 24 h in Fig. 10, both being 95%, are the result of the loss of one of the 20 leaves used for each treatment of this experiment.) Although the variability in this small scale experiment was rather large, it can be seen, especially from Fig. 11, that some kind of change must have been induced by the first dose resulting in a mechanism, which reaches a maximum after approx. 12 hours and gradually breaks down in the following days, although it is still noticeable

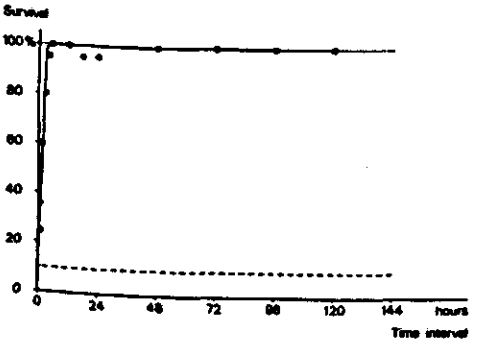


Fig. 10. Effect on survival of two semi-lethal X-ray doses (3 krad; 200 rad/min) given at various time intervals. An acute dose of 3 krad X-rays gives 100% survival (.....) and a dose of 6 krad gives 10% survival (---).

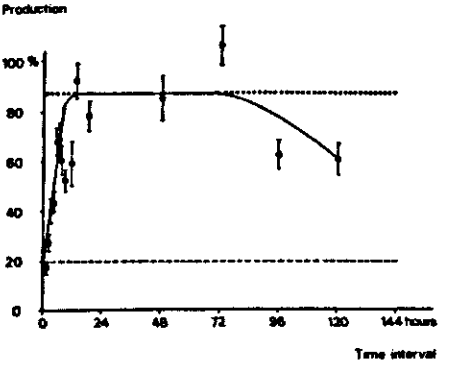


Fig. 11. Effect on production of two semi-lethal X-ray doses (3 krad; 200 rad/min) given at various time intervals. An acute dose of 3 krad X-rays gives 87% production (.....) and a dose of 6 krad gives 20% production (---).

after 5 times 24 hours. (Genetic effects: see section 3.3.2). This conclusion is drawn from the fact that two doses of 3 krad, separated by for instance 24 hours, have exactly the same effect, as it is drawn in Fig. 11, compared with only a single dose of 3 krad. One would have expected that the second 3 krad would have added at least the same amount of damage to that of the first dose (13% decrease in production, measured approx. 6 months after the irradiation) or probably even more. Instead, no additional damage was observed, between an 18-h and a 72-h interval, if the form of the curve in Fig. 11 is correct.

The form of the curve, as it is drawn in Fig. 11, is based upon a number of considerations, rather than upon the fairly variable data. One is the comparable form of the curve for survival (Fig. 10), which shows a striking increase to 100% and stays that way from approx. 12 to 120 hours. It indicates that after a 12-h interval the second 3 krad had no effect. Whether the 2nd dose also effected the 1st dose cannot be concluded from Fig. 10 since values above 100% are not possible with survival. This, however, could have been considered in Fig. 11 where a few data are above the 3 krad line. The distance to that line, however, was not significant and moreover it seems unlikely that the second dose was of influence upon the permanent damage of the first dose after an interval of for instance 72 hours. Most stages of radiation action in an aqueous system are extremely short, such as the physical stage, during which the energy absorption takes place (10^{-13} sec), the physico-chemical stage, during which intermolecular energy transfer in excited and ionized molecules takes place (10^{-10} sec) and the chemical stage, where intramolecular energy transfer as well as secondary reactions take place (10^{-6} sec). The final one, the biological stage, may take seconds to hours or much longer, depending on the alteration studied (metabolic alteration, tumors, cell death, hereditary alterations), on the system irradiated and on the environmental conditions (storage in liquid nitrogen for instance) (Dertinger & Jung, 1969; Platzman, 1958).

Comparable experiments strongly suggested that an interval of 8-10 times 24 hours would have resulted in an equally large effect of the two fractions compared with an acute dose of 6 krad. In Fig. 14, for instance, the two fractions (500 rad X-rays as initial dose and 6 krad X-rays as second dose) are equally effective, either as an acute dose of 6,5 krad or when separated by 168 hours and up. A similar result was obtained with fast neutrons (see Fig. 15).

From these considerations and the form of the curves in figs. 10 and 11

it is concluded that the main effect was caused by the first irradiation which induced a mechanism that prevented a second irradiation of 3 krad X-rays having any effect when applied after a given time interval. (Later in this section we will see that the extent of the protective effect after a single pre-treatment happens to be approx. 3 krad (X-rays), which may be one of the origins of the confusion about the explanation of figs. 10 and 11).

Whether this effect is the result of an improved radio-induced repair mechanism or of a radio-induced protection is not known. Protection implies prevention of part of the damage, either by competitive protection or by restitutive protection. Competitive protection refers to the ability of certain chemical compounds to compete with the biomolecules for the diffusible radicals. They are called radical scavengers, with the characteristic that the contribution of the indirect effect is reduced. Restitutive protection does not alter the number of primary lesions but part of the damage is restituted by the protective agent.

The term "repair" generally refers to enzymatic processes which are under genetic control. As has been discussed in Section 1.1, several types of repair mechanisms, each with a distinct repair time class and linked to particular enzyme activities are recognized. In this context also the word "reactivation" is used, which refers to the consequences of repair (the actual molecular process of the elimination of damage), such as in increase in survival-rate.

Since the words repair, protection and reactivation are used inconsistently in the literature and also because the mechanism involved in my material was not investigated and therefore not known, it has been decided to use the word "protection" to describe the phenomena after fractionated irradiations.

Many publications can be found describing similar phenomena, which are usually attributed to protective mechanisms, in various organisms such as *Saccharomyces* (Maisin et al., 1960), chicken (Christian et al., 1965), dogs (Dimitrow, 1964; Graevskaja & Kejlina, 1956; Ainsworth & Leong, 1966; Page et al., 1965), hamster (Holloway et al., 1968; Littlebrand et al., 1967), mice (Cronkite et al., 1950; Hoffman et al., 1965; Sacher & Grahn, 1957; Yuhas, 1968), monkeys (Paterson et al., 1956; Paterson & Matthews, 1952), pigs (Chaput & Kovacic, 1970; Nachtwey et al., 1967; Page et al., 1965), rabbit (Leidl & Zankl, 1970; Ramsell & Berry, 1966), rats (Braun, 1957; Braun, 1962; Taenzer & Krokowski, 1966) and sheep (Page et al., 1965).

Generally whole animals are used to study the effect of one or more pre-treatments (called initial dose, primary dose or conditioning dose in different publications) prior to a second total body (mass) irradiation after

various time intervals. Survival or mortality is mostly used as the parameter, for instance by Christian et al. (1965) with chicken. Some authors irradiated only part of an animal, such as the skin of pigs, using skin reactions as the parameter (Bewley et al., 1967). Others use repopulation as the parameter, for instance in mice (Denekamp et al., 1966; Emery et al., 1970).

A number of authors have tried to review all the data on radio-induced protection and to classify the many different and often confusing observations obtained by various scientists (Dacquisto, 1959; Maisin et al., 1960; Krokowski & Taenzer, 1966; Taenzer & Krokowski, 1968). Krokowski (1968) standardized the results, comparing the LD_{50}'s of the radiation effects with and without pre-irradiation by putting them in a three-dimensional co-ordinate system.

From the data obtained from the literature a number of general conclusions can be drawn about the effect of dose fractionation upon animals or animal tissue, namely:

1. pre-irradiation induces a protective mechanism;
2. this protection depends upon the intensity of the initial dose, the optimum being approx. 10-20% of the LD_{50};
3. the protection also depends upon the time interval between first and second dose; generally a time interval of 10-14 days is required for optimum protection; the protection can last several weeks or even months;
4. a repeated pre-irradiation is less effective than a single initial irradiation.

A peculiar and interesting fact is reported by one author (Krokowski, 1968), namely that radio-induced protection is transmissable by injecting the serum from pre-irradiated animals into non-irradiated ones. These animals showed a striking increase in radioresistance (for similar experiments in *Saintpaulia*, see p. 51 and 52).

In plants most dose fractionation investigations have included the phenomenon of chromosome aberrations (Davies, 1962; Lane, 1952; Sax & Luippold, 1952). All these experiments confirm the conclusions drawn from previous experiments that fractionation of an acute X-ray dose always reduces the yield of chromosome reunions, usually to that of the sum of the yield of the (equal) fractions. Lane (1952), using *Tradescantia* microspores, found after an interval between the two fractions of 4 hours a 50% breakage compared with the control. However, the same amount of breakage, compared with the control, was found after an interval of 12 hours and again a reduced breakage after an interval of 18 hours. Lane concluded that radiation affects the initial sensitivity of the cell to

chromosome breakage, but could not explain the decreased yield of aberrations after an 18-h interval. Davies & Wall (1960) studied the V^{by} locus of *Trifolium repens* and found that either fractionation or chronic irradiation were always less effective than the acute total dose. In terms of the frequency of mutations they concluded that acute irradiation is more effective. Davies (1962) again working with white clover, studied the effect of a low initial dose on the protection induced. He found that at 25°C protection was built up rapidly and reached a maximum after approx. 8 hours and then decreased. At 8°C (24 hours before, during and 24 hours after the treatment) the protection did not decrease, not even after 4 days. Furthermore the protection was dependent on the presence of oxygen; in nitrogen no protection was observed. Ancel (1928) studied the effect of fractionation on *Vicia faba* seedlings measuring the height of the plantlets 4 days after an acute dose and various fractionation treatments, applying intervals ranging from 1 to 24 hours. Fractionation always yielded higher plantlets than an acute dose. Sybenga (1964), using sectorial discoloration of the first leaf of *Crotalaria* found, unexpectedly, that fractionation yielded less than twice the effect of a half dose, which points towards a protective effect of the first dose. Kaplan (1951) reported, in contrast to the data mentioned before, an increase in sterility after fractionated irradiation of dormant barley seeds compared with an acute dose. The sterility of the plants also increased with increasing time interval. Gene mutations did not react upon fractionation.

Most authors, mentioned before, have worked with complex heterogeneous systems, such as root tips, seedlings and plants. The disadvantage of such a system was demonstrated by Miller & Colaiace (1970), who showed, using *Vicia faba* roottips, that there was a heterogeneity of the cells to radiosensitivity and time. Consequently the yields of rings and dicentrics varied with fixation time and time of irradiation during G_1 so that two-hit chromosome aberrations do not reliably interpret the results of dose-fractionation.

From the results, obtained in the first experiment mentioned before, and from figs. 10 and 11, various questions arise:

1. Can an "optimum" initial dose be determined which induces a maximum protective effect in the leaves?
2. What is the relationship between protection and the time interval separating the initial dose and the second dose?
3. What is the extent of the protection?
4. What is the effect of a repeated irradiation using the "optimum" initial

dose separated by the "optimum" time interval?
5. What is the relationship between the various factors and the mutation frequency?

3.3.1.1 Initial dose

A variety of terms is used in literature to distinguish the first dose from the next one(s) in a dose fractionation or split dose experiment. They range from "initial dose", "pre-irradiation" and "pre-treatment" to "primary dose", "first fraction", "the first of the split doses" and "the conditioning dose".

Its use depends not only on personal preference but also on the material used in the experiments, the latter terms mentioned mainly being used in medically orientated investigations. I prefer "initial dose", replacing it by "pre-irradiation" or "pre-treatment", when appropriate.

Various initial doses have been applied, ranging from very small ones (1, 10, 30, 75 and 150 rad X-rays and comparable rad doses of fast neutrons) up to 3 krad X-rays or fast neutrons. These were followed by a series of second doses, namely the semi-lethal dose of 3 krad X-rays, the sub-lethal dose of 6 krad and a number of medium and lethal doses (4, 5, 7, 8, 9, and 10 krad X-rays) or comparable fast neutron doses. The high second doses were given to test the extent of the protection.

Since no symptoms have been observed that indicate a relation between the size of the second dose and the time interval, it has been decided to apply a comparatively short interval between first and second dose, namely 8 hours. This gives (almost) maximum protection and has been defined, in Section 3.3.1.2, as the "optimum" time interval. The choice was partially conditioned by practical reasons (most of the fractionation experiments being practicable within one working-day) and by scientific reasons (a better comparison with the repeated irradiations, which had to have 8-h intervals; see Section 3.3.1.2).

As can be seen in figs. 12 and 13 (survival and production, respectively, after various initial doses X-rays or fast neutrons) a very pronounced protective effect is induced by an initial dose of 250-1000 rad of X-rays of approx. 500 rad using fast neutrons.

Over the optimum initial dose-range the effect of the second dosage of 6 krad is greatly reduced (Fig. 12). The 6 krad X-ray dose, used alone, would have given a survival of about 20%. When used as a second dose the survival is

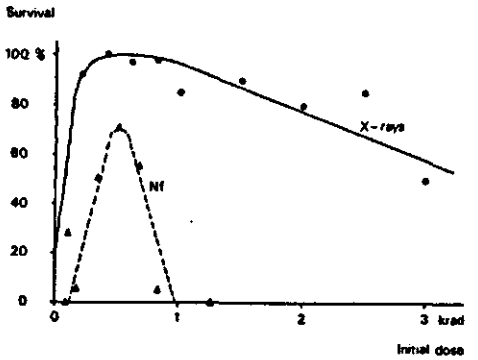


Fig. 12. Effect of various initial doses on survival. Second doses were 6 krad X-rays (200 rad/min) (——) or 3.3 krad fast neutrons (17 rad/min) (---); there was an 8-h interval between first and second dose.

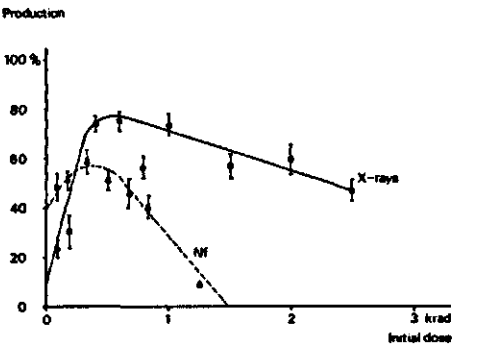


Fig. 13. Effect of various initial doses on production. Second doses were 5 krad X-rays (200 rad/min) (——) or 2.5 krad fast neutrons (17 rad/min) (---); there was an 8-h interval between first and second dose.

almost 100% when the first dose was around 500 rad. Thus the effectiveness of the second dose on survival has been reduced by a factor of 5. At the highest initial dose used, 3 krad, the effect of the second dose of 6 krad (for a total dose of 9 krad) is also substantially reduced, here by a factor of about 2.5. It is further interesting to note that a single dose of 9 krad would normally be completely lethal and produce no survival.

An exact optimum of the initial dose for X-rays is hard to define since a fairly large dose range, covering a few hundred rads, induces (close to) maximum protection. With fast neutrons the optimum initial dose seems to be approx. 500 rad. Moreover the impression is obtained that the optimum seems to depend on the extent of the second dose: a larger second dose often requires a larger initial dose for maximum protection but is not always conclusive. This indicates a (small) increase in protection with increasing initial dose in the dose range mentioned before. This phenomenon is demonstrated when repeated initial doses are applied: see Fig. 23, comparing 5 krad Nf with lower last doses. However, the unrepaired damage of the increasing first dose interferes with the protection that increases less. They mask the effect of the protection, especially when higher initial doses are applied, and cause a diffuse optimum, especially in the case of X-rays.

The somewhat arbitrary choice of the "optimum" initial dose has therefore fallen on an initial dose in the lower region of the dose range, namely 500 rad X-rays or fast neutrons. This dose minimizes the effect of the first dose and induces (close to) maximum protection.

Unfortunately some preliminary experimental results suggested that 170 rads of fast neutrons was the optimum initial dose in African Violet instead of 500 rad. This means that a few fast neutron experiments have been carried out with a "sub-optimum" initial dose, e.g. repeated irradiations, which consequently cannot directly be compared with the repeated X-ray irradiation using the "optimum" initial dose (see Section 3.3.1.4).

After the definition of the optimum initial dose, the second question had to be solved, namely which is the "optimum" time interval.

3.3.1.2 Time interval

Various intervals, ranging from 1 to 12 hours and at 24-h intervals thereafter up to 10 times 24 hours, have been applied to separate the initial dose (500 rad X-rays or 170 rads of fast neutrons) from a second dose (generally 3 krad X-rays or 1700 rads of fast neutrons and 6 krad X-rays or 3400 rads of fast neutrons).

As is shown in figs. 14 and 15 it is evident that a protective mechanism builds up very rapidly, reaching its maximum at about 8-12 hours and gradually decreasing with increasing interval. After approximately 7 x 24 hours the protective effect has disappeared completely, regardless of the type of radiation used. There is a marked difference between the form of the curves in figs. 14 and 15 compared with figs. 10 and 11. The decrease in survival and production is none or much slower with increasing time interval in figs. 10

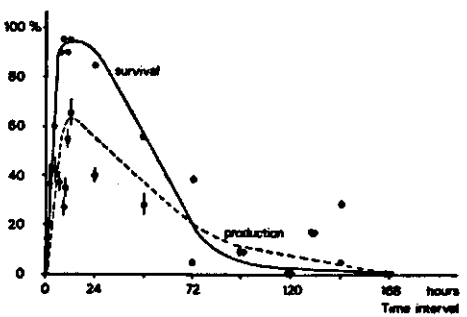


Fig. 14. Effect of various time intervals on survival and production at an initial X-ray dose of 500 rad and a second X-ray dose of 6 krad. At 6 krad there is approximately 10% survival and approximately 20% production; an X-ray dose of 6.5 krad is lethal. Dose rate was 200 rad/min.

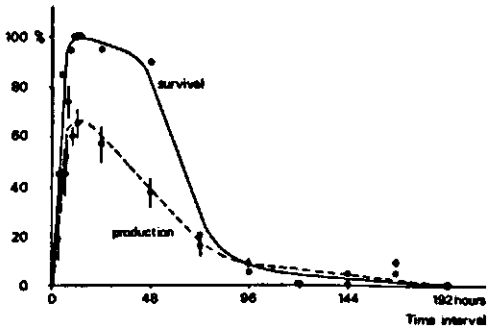


Fig. 15. Effect of various time intervals on survival and production at an initial fast neutron dose of 170 rad and a second fast neutron dose of 3.4 krad; fast neutron doses of 3.3 and 3.5 krad are lethal. Dose rate was 17 rad/min.

and 11 than in figs. 14 and 15: after 72-96 hours both survival and production are below 10% in contrast to the high %'s in figs. 10 and 11 (100% survival and approx. 87% production).

This may be the result of a somewhat better protective capacity by the high(er) initial dose in figs. 10 and 11 (3000 rad) (see also Section 3.3.1.1) but probably is mainly to be attributed to the higher second doses used in figs. 14 and 15, which has tested the protection much more seriously.

Figures 14 and 15 moreover indicate that after a relatively long interval (168 hours and up), the effect of two fractions (500 and 6000 rad) is equal to that of the total acute dose (6500 rad). Consequently, the effects of the two fractions are completely cumulative. This is evident because the lines for survival and production otherwise would have gone back to a point between the lethal point and their respective base lines (for survival 10% and for production 20%). In this case fractionation does not result in a lower effect, provided the right time interval is chosen. Although the system used may have something to do with it (no cell division during the whole experiment and thus no cell elimination) one wonders whether the statement in the beginning of Section 1.2, page 8 ("generally the biological effect of fractionation is less .....") would still hold true if experiments reported in the literature had been carried out with more different and longer time intervals.

In relation to the repeated irradiations planned (Section 3.3.1.4), the "optimum" time interval was defined as the shortest time needed for maximum protection. The interval of 8 hours was therefore selected and permitted 60 repeated irradiations of the material with the optimum initial dose within a 20 day period. Intervals of 12 hours would have extended the experiments to 30 days which is too long for the separated leaves to remain in sealed plastic bags without a considerable loss of vitality.

3.3.1.3 Extent of protection

To test the extent of the radio-induced protection a series of second doses, the highest of which are lethal when given as an acute single dose, were applied. As can be seen in Figure 16 even after an initial dose of 600 rad X-rays (or 500 rad fast neutrons) and a second dose of 9 krad X-rays (or 4.5 krad fast neutrons) there is a surviving fraction which corresponds with an acute single dose approximately 3 krad X-rays or 1.5 krad fast neutrons lower. In other words: a comparatively low initial dose of 500-600 rad induces a protection equivalent to approximately 3 krad X-rays or 1.5 krad fast

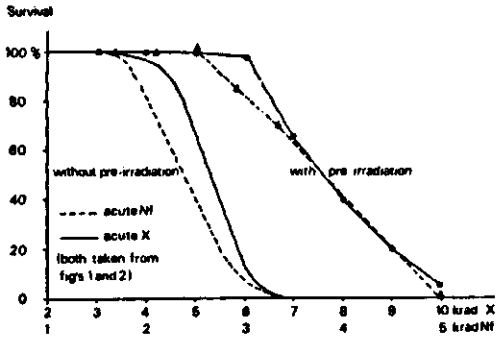


Fig. 16. Extent of protection induced by an initial X-ray dose of 600 rad (—●—) or an initial fast neutron dose of 500 rad (--▲--), tested by applying various second doses of X-rays or fast neutrons after an 8-h interval. Dose rate was 200 rad/min (X-rays) and 17 rad/min (fast neutrons).

neutrons.

This protection is also demonstrated by an experiment in which an optimum initial dose (500 rad X-rays) or the "sub-optimum" fast neutron dose of 170 rad preceeded a series of second doses, either fast neutrons or X-rays, after an 8-h interval. Thus, an initial X-ray dose was followed either by a second X-ray dose or by a second fast neutron dose. Similarly, initial doses of fast neutrons were followed by a second dose of either fast neutrons or X-rays.

The data show (figs. 17 and 18) that the distance between the acute lines and the other parallel lines is equal to approximately 3 krad X-rays or 1.5 krad fast neutrons. Looking at Fig. 17 another way we can see, as before, that

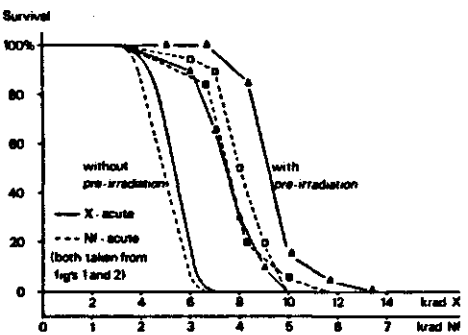


Fig. 17. Effect on survival of the (optimum) initial dose (500 rad of X-rays or 170 rad of fast neutrons) after various second doses of X-rays or fast neutrons. -Δ-, both initial and second doses of X-rays, -▲-, initial dose of X-rays, second dose of fast neutrons, --■--, both initial and second doses of fast neutrons; --□--, initial dose of fast neutrons, second dose of X-rays. Dose rate was 200 rad/min (X-rays) or 17 rad/min (fast neutrons).

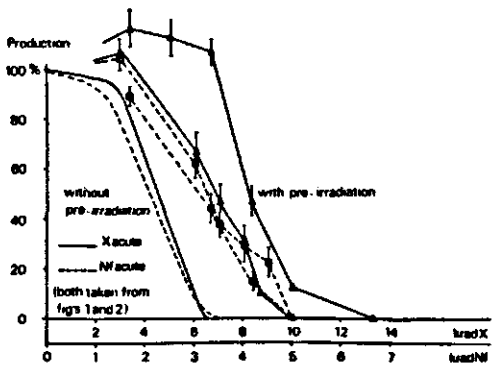


Fig. 18. Effect on production of the (optimum) initial dose (500 rad of X-rays or 170 rad of fast neutrons) after various second doses of X-rays or fast neutrons. -Δ-, both initial and second doses of X-rays; -▲-, initial dose of X-rays, second dose of fast neutrons; --■--, both initial and second doses of fast neutrons; --□--, initial dose of fast neutrons, second dose of X-rays. Dose rate was 200 rad/min (X-rays) or 17 rad/min (fast neutrons).

at 6 krad of an acute single dose there is only ± 10% survival. When 6 krad is the second dose, survival is raised to approx. 100%. For production (Fig. 18) the figures are somewhat lower, except in the case of X-rays preceeding fast neutrons where a pronounced stimulation can be noted. The origin of this, not significant, stimulation is not known and relates to a similar phenomenon which has been discussed in Section 3.3.1. There the question was raised whether the second dose influences the repair of part of the effect of the first dose. However, if the "X-Nf line" in Fig. 18 is drawn correctly, it means more than the (even complete) repair of the small first dose: in addition a stimulation of one kind or another has been induced which results in a production above the 100% of the control.

In any case one important conclusion can be drawn from figs. 17 and 18, namely that both X-rays and fast neutrons are equally effective in the induction of a comparatively equally large protection against a second dose of radiation, either X-rays or fast neutrons.

3.3.1.4 Repeated irradiation

To investigate whether a repeated irradiation with the optimum initial dose, separated by optimum time intervals would result in an increased, decreased or equal protection, 500 rad X-ray or 170 rad fast neutron doses were repeatedly applied at 8-h intervals. During the treatment, the leaves, sealed in plastic bags, were kept in the dark except when handled and irradiated. The temperature was kept at approximately 20°C; the effect of other temperatures has not been studied.
After 1, 5, 10, 15 etc. up to 60 repetitions a series of last doses (1.5, 3, 4.5, 6, 8, and 10 krad X-rays or 0.8, 1.7, 2.5, 3.4, 5 and 6.8 krads of fast neutrons) were applied to test the protection. A decrease would mean that a single pre-treatment yields the maximum possible protection value which disappears in time, independent of subsequent irradiations. A steady level would indicate that the protection is kept maximum after the single pre-irradiation and stays that way until 60 repetitions, during which every irradiation compensates for the decrease, which goes on continuously. An increase would indicate that every irradiation adds some (the same amount?) protection to the system, which is bound to reach a maximum when the decrease in protection, induced by the first irradiations, sets in.

It can be seen from Fig. 19 that the protection increases rapidly: a last dose of 10 krad X-rays, being lethal after a single pre-irradiation (the "X-X

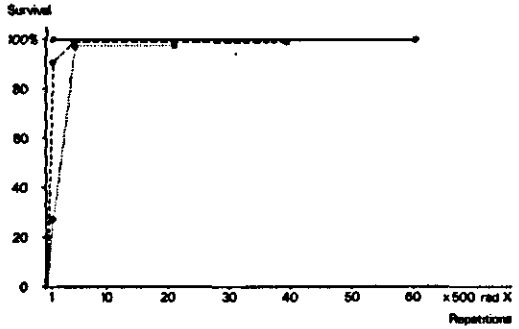


Fig. 19. Effect on survival of repeated X irradiations of 500 rad at 8-h intervals; various last doses of X-rays were applied, namely 6 krad (___), 8 krad (---) or 10 krad (.....). Dose rate was 200 rad/min.

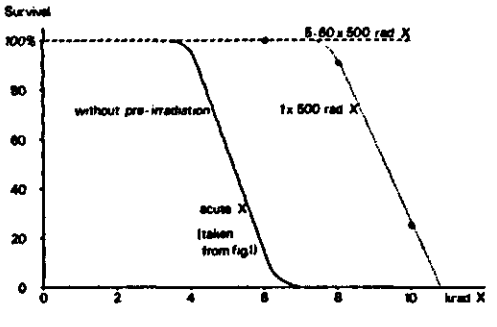


Fig. 20. Effect on survival of repeated X irradiations of 500 rad at 8-h intervals; various last doses were applied. Dose rate was 200 rad/min.

lines" in figs. 17 and 18) results in 100% survival after 5 repetitions with the optimum initial dose of 500 rad X-rays and stays that way until 20 repetitions.

In Fig. 20, which is based on the same data as Fig. 19 but presented differently, an impression about the extent of the protection can be obtained. The distance between the "acute line" and the "one pre-treatment line" is approx. 3-4 krad, whereas the distance between the latter and the following one (5 repetitions) is probably about the same. The X-ray data, however, are incomplete because the series of high last doses was not long enough.

With fast neutrons the range of last doses was sufficiently long to determine the increasing protection with increasing number of repetitions. As is shown in Fig. 21 maximum protection is obtained after 10 repetitions. (In fact the "10 x 170 rad line" in Fig. 21 represents the lines for 10, 15 and 20 repetitions, which were almost identical. The exact optimum number of repetitions will be defined and discussed later.) The distance between the

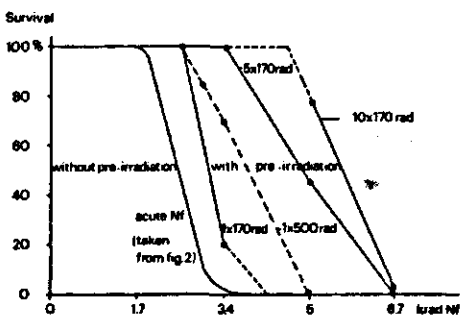


Fig. 21. Effect on survival of repeated fast neutron irradiations of 170 rad at 8-h intervals; various last doses were applied. The "500 rad line" is taken from Fig. 16. Dose rate was 17 rad/min.

"10 x 170 rad line" and the acute line is approx. 3 krad, which is substantially more than after only one pre-treatment. But we must not forget that the initial dose of 170 rad fast neutrons used for the repeated irradiations, has induced a smaller protection after only one pre-treatment as was the case with an optimum initial dose (see Fig. 16). It might therefore take a greater number of repetitions to reach maximum protection when using a sub-optimum dose. This would explain the relatively small distance between the acute line and the "1 x line", and the relatively large distance between the "1 x line" and the "5 x line". The, again small, distance between the "5 x line" and the "10 x line" indicates a saturation point, where the protection has reached its absolute maximum value, following the type of repeated irradiation applied. (Other doses separated by shorter (?) time intervals might result in a higher (?) maximum value.)

Figures 22 and 23 (survival and production, respectively, after repeated irradiations with 170 rad fast neutrons) show that the protection reaches a maximum after approx. 15 repetitions and then decreases fairly rapidly. A dose of 5 krad fast neutrons, for instance (solid line, closed circles), applied after 15 repetitions (with 170 rad fast neutrons) results in approx. 90% survival (Fig. 22) and 40% production (Fig. 23). After only a single pre-treatment these figures are just about 0%, both for a pre-irradiation with 170 rad fast neutrons (Fig. 18, the Nf-Nf line) and for a pre-irradiation with the optimum initial dose of 500 rad fast neutrons (Fig. 16).

From these figures it would appear that fast neutrons are less effective

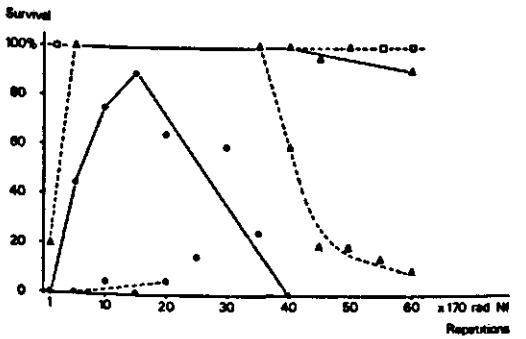


Fig. 22. Effect on survival of repeated fast neutron irradiations of 170 rad at 8-h intervals. Last doses of fast neutrons were 6.7 krad (--o--), 5 krad (--•--), 3.4 krad (--Δ--), 1.7 krad (--▲--), 170 rad (--□--). Dose rate was 17 rad/min.

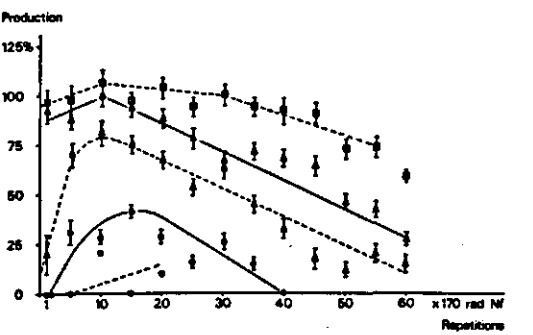


Fig. 23. Effect on production of repeated fast neutron irradiations of 170 rad at 8-h intervals. Last doses of fast neutrons were 6.7 krad (--o--), 5 krad (--•--), 3.4 krad (--Δ--), 1.7 krad (--▲--), 170 rad (--□--). Dose rate was 17 rad/min.

in the induction of protection than X-rays. (5 krad fast neutrons as a last dose never results in 100% survival (Fig. 22), whereas 10 krad X-rays as a last dose reaches 100% survival after 5 repetitions and stays that way until 20 repetitions (Fig. 19)). It seems to be confirmed by Fig. 12, where the maximum of the 3.3 krad fast neutron dose stays below that of the (somewhat less heavier) 6 krad X-ray dose.

The impression, from figs. 22 and 23 (fast neutrons) compared with figs. 19 and 20 (X-rays), that fast neutrons are less effective in the induction of protection does not agree with the results presented in figs. 17 and 18. As has been discussed in Section 3.3.1.3 the four lines (X-X, X-Nf, Nf-Nf and Nf-X) are completely comparable with each other, especially when the "Nf-Nf-line" (the lowest of the four) is replaced by the "Nf-line" of Fig. 16 (a similar treatment, but with the optimum initial dose of 500 rad fast neutrons). This line is closer to the average of the three others and therefore better fits the conclusion in Section 3.3.1.3 that neutrons are equally effective compared with X-rays in inducing protection.

The impression, after repeated irradiation, that neutrons are less effective is probably the result of the use of a sub-optimum (initial) dose of 170 rad which becomes more apparent after repeated irradiation than after a single pre-treatment.

The observation that maximum protection is obtained after 10-15 repetitions (figs. 22 and 23) seems logical. The effect of the first irradiation with the initial dose starts to break down substantially after 2-3 days, as can be seen in Fig. 15. This rapid breakdown of the protection, induced by the first dose(s), sets in after 6-9 repetitions (2-3 days) and apparently is hardly compensated by the (probably decreasing) additions to the protection by the subsequent doses. The final result is a maximum that is reached a restricted number of repetitions later. From thereon the protective value slowly decreases as the result of the increasing extent of the unrepaired damage or of the decreasing protective value, or of a combination of the two. The foregoing is based upon results with fast neutrons and probably also holds true for X-rays. The last doses with X-rays were, unfortunately, not high enough (Fig. 19) to prove this assumption.

It would have been an interesting experiment to irradiate repeatedly, up to 15-20 times for instance, and then test the extent of the protection by the irradiation with high last doses at the same periods as used in my experiments. One wonders whether the lines would decrease earlier (faster) or later (slower).

Earlier and/or faster would indicate that the protection decreases earlier or more rapidly because the lacking repetitions do not add protection anymore. Later and/or slower would indicate that the protection reaches a stable value by a given number of repetitions and stays that way because it is not anymore damaged by subsequent irradiations, whereas also no unrepairable damage is added to the system anymore.

3.3.1.5 Conclusive remarks

The data presented above agree in part with the data in the literature, using animals or animal (mammalian) tissues, listed in Section 3.3.1. The optimum initial dose mentioned there is approx. 15% of the LD_{50} and the extent of the protection induced (increase in tolerance by ca. 1.7) seems to fit fairly well with the data presented here. In contradiction, however, is the fact that the protection is reported to stay active in animals over an extremely long period whereas in African Violet most of the protective effect has disappeared after 5 days in the case of a single pre-treatment. Also in contradiction is the fact that in animals a single pre-irradiation induces a better protection than a repeated one. The data presented here clearly demonstrate a maximum protection after a 10-15 fold repetition of the pre-irradiation dose.

The comparison of African Violet with animals or animal tissues is not justified. Animal cells react differently from plant cells. The partly agreeing results, reported in the literature, are, however, striking enough to justify further investigations with animals or animal (mammalian) tissue, in particular with relation to radiotherapy. Some scientists, however, are not convinced by the literature reviews mentioned in Section 3.3.1, in my opinion as the result of the incompleteness of many split dose experiments, in which generally a very restricted number of doses are separated by a sometimes restricted and arbitrary number of different time intervals. As I have seen with my material, it is of utmost importance to choose the right first and subsequent doses and the right series of time intervals to demonstrate the effects of the first dose. A wrong choice would have given confusing and unexplicable results.

In connection with the results of Krokowski (1968), who reported a transmissable radio-induced protection (Section 3.3.1) I tried to investigate whether this phenomenon also takes place in African Violet. Grafting (irradiated) leaves on an unirradiated plant turned out to be impossible, so the experiments were discontinued. It would, however, be interesting to carry out a number of experiments to further investigate this phenomenon such as to irrad-

iate one half of a complete plant with 500 rad for instance and to check the radioresistance of leaves of the unirradiated part, after given time intervals. Another method would be to irradiate the leaf disc of a detached leaf and to test the radioresistance of the leaf petiole by irradiating it with a series of high doses after a given time interval. Both experiments may bring to light the existence of a transportable protection within a plant (part). If so, one could try to transfer the component(s) responsible by pulping irradiated leaves and extracting the juice with a subsequent uptake of the extract by detached untreated leaves.

In plants, as has been discussed in Section 3.3.1, very few dose fractionation studies have been reported. Just before the final version of this study Horsley and Laszlo published results of dose fractionation experiments with algae (1971). They reported an unexpected additional recovery after a first X-ray dose, using a synchronous cell culture of *Oedogonium*. A sharp rise in survival was obtained in a split dose experiment (first dose 1200 rad X-rays, second dose 4200 rad X-rays) after a 2-h interval, reaching a maximum between 6-8 hours before falling steeply. The explanation suggested was an efficient recovery mechanism, set into play by the first dose of radiation.

3.3.2 Genetic effects

As has been said before (Section 2.3) the third parameter, expressed as mutation frequency (number of mutants per 100 plantlets) or as the number of mutants per 100 irradiated leaves, is the least reliable. The reason being the long time between the irradiation and the observation of the mutants (9-10 months), during which many things may happen to part of the experiment. Another reason is that, especially after heavy treatments, it is difficult to decide whether a plantlet grows slowly or abnormally because of either genetic or physiological disturbances. Moreover, the small number of plantlets obtained after heavy treatments, results in a poor significance of the figures for mutation frequency, reflected in the far from ideal form of the curves in figs. 24, 27 and 29. Fortunately, most experiments included a range of second doses (in the case of a single pre-treatment) or a range of last doses (in the case of repeated irradiations). The procedure to obtain the best possible results, using this parameter, was as follows: to eliminate the defects in the data of the individual lines the average of all second or last doses was calculated. Every single line was then compared with the average line and the ones that fitted the general tendency best was selected for the calculations to determine

the number of mutants per 100 irradiated leaves, in which all three parameters were discounted. The conversion from one figure to the other, such as Fig. 24 into parts of figs. 25 and 26, thus gives an impression of the effect of fractionation upon the genetic parameters. No absolute value should therefore be attached to the lines. On the other hand, the persistent tendency obtained, makes it plausible that the truth has not been violated too much.

In Fig. 24 the effect of *initial dose* of either X-rays or fast neutrons upon the mutation frequency is shown. As the RBE, for survival and production, was 1 (figs. 12 and 13), I have expressed the mutation frequency on the same basis. The abscissa of the graph therefore gives the initial dose for either X-rays or fast neutrons.

With increasing initial dose, an increasing protection against genetic effect builds up, reaching a maximum for X-rays around 1.5 krad and for fast neutrons between 0.5 and 1 krad, reflected in minimum mutation frequencies. (The first minima, at an initial dose of approx. 150 rad, have been neglected because the low survival and production in that case result in a very poor significance of the mutation frequency among the few plantlets produced.) With still increasing dose the protection seems to stay relatively unchanged for X-rays, but decreases rapidly for fast neutrons.

With still increasing initial dose the protection stays relatively unchanged for X-rays.

When the results in figs. 12 and 13 are compared with those in Fig. 24 maximum protection is found around an initial dose of 500 rad, resulting in maximum survival and production. For mutation frequency maximum protection, resulting in minimum mutation frequency, seems to be shifted towards a higher initial dose, especially for X-rays.

Furthermore the more drastic reaction with fast neutrons rather than X-

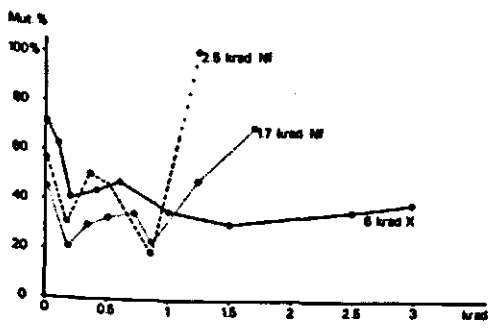


Fig. 24. Effect of various initial X-ray or fast neutron doses on mutation frequency. Second dose was 6 krad X-rays or 1.7 and 2.5 krad fast neutrons respectively; 8-h intervals between first and second dose were applied. The scale of initial doses applies to both X-rays and fast neutrons on the basis of an RBE is 1. Dose rate was 200 rad/min (X-rays) or 17 rad/min (fast neutrons). (The highest point of the "2.5 krad Nf-line" is very questionable.)

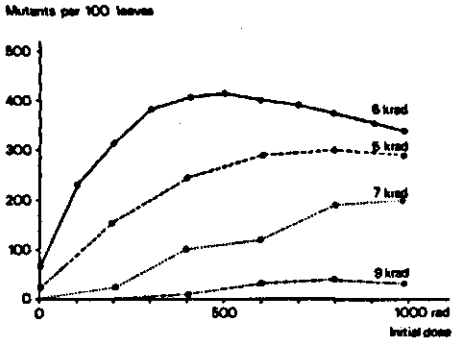


Fig. 25. Number of mutants per 100 leaves after various initial and second doses of X-rays. Dose rate was 200 rad/min. The interval between first and second dose was 8 hours.

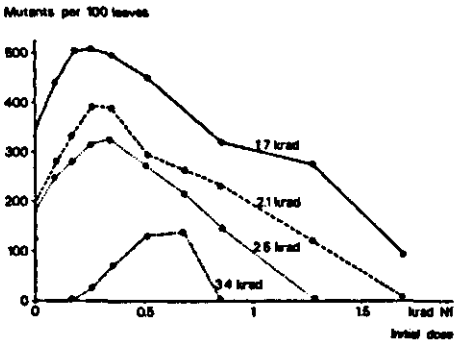


Fig. 26. Number of mutants per 100 leaves after various initial and second doses of fast neutrons. Dose rate was 17 rad/min. The interval between first and second dose was 8 hours.

rays is striking in all three diagrams (representing the three parameters used) so that identical or almost identical (biochemical) reactions may be the origin of the correlation between the behaviour of the three parameters.

For both X-rays and fast neutrons the number of mutants per 100 leaves was calculated as mentioned before. The result is shown in figs. 25 and 26, which demonstrate, that 500 rad X-rays, in combination with 6 krad X-rays as a second dose and after an 8-h interval, produces the maximum number of mutants per 100 leaves, namely 415, being 163% of the corresponding optimum acute dose of 3 krad X-rays. An initial dose of 250 rad fast neutrons, in combination with a second dose of 1.7 krad fast neutrons, produces the highest number of mutants per 100 leaves ever obtained, namely 505, which is 146% of the corresponding optimum acute dose of 1.5 krad fast neutrons. (With the number of mutants per 100 leaves as parameter, the RBE values for fast neutrons would again be approx. 2, and not 1, as for survival and production as parameter.)

The effect of *time interval* upon mutation frequency, as is shown in Fig. 27, behaves in a similar way: a rapidly increasing protection with still larger intervals, which was already demonstrated for survival and production in figs. 14 and 15. Consequently a maximum number of mutants is to be expected just before survival and production reaches a maximum. This is demonstrated in Fig. 28 where 500 rad X-rays, in combination with 6 krad X-rays, produced the maximum number of mutants per 100 leaves after a 10-h interval. The absolute number, 240, however, is low in comparison with the corresponding optimum acute dose of 3 krad (270) as well as compared with the initial dose experiments, described just before (415). Since the whole 6 krad line is lower than

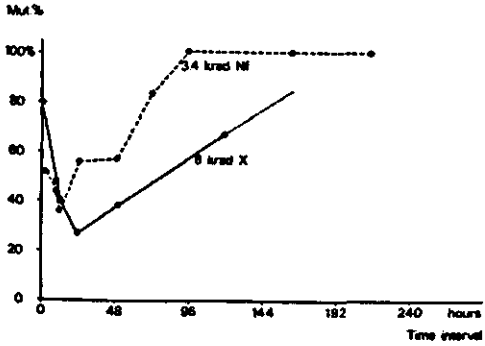


Fig. 27. Effect of various time intervals on mutation frequency. Initial dose was 500 rad X-rays or 170 rad fast neutrons; second dose was 6 krad X-rays or 3.4 krad fast neutrons respectively. Dose rate was 200 rad/min (X-rays) or 17 rad/min (fast neutrons).

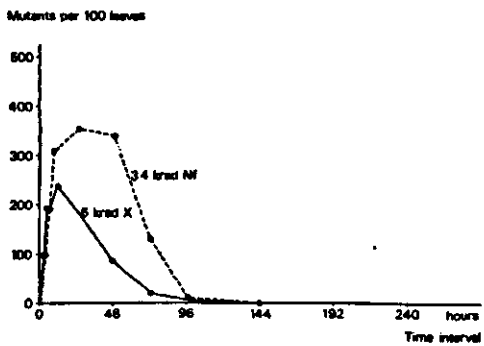


Fig. 28. Effect of various time intervals on number of mutants per 100 leaves. Initial dose was 500 rad X-rays or 170 rad fast neutrons; second dose was 6 krad X-rays or 3.4 krad fast neutrons, respectively. Dose rate was 200 rad/min (X-rays) or 17 rad/min (fast neutrons).

expected, the only possible explanation is that this second dose had a more damaging effect than usual, for reasons unknown.

The conclusion, namely that the optimum time interval approximately lies around 10 hours, however, is valid.

With fast neutrons a similar picture was obtained: a high number of mutants between 8 and 48 hours with a maximum at a time interval of 24 hours. An initial dose of 170 rad, in combination with a second dose of 3.4 krad fast neutrons, produced 355 mutants per 100 leaves, being 142% in comparison with the corresponding acute dose of 1.5 krad fast neutrons.

The effect of *repeated irradiation* with the optimum initial dose, separated by the optimum time interval, is shown in Fig. 29 for X-rays. Only the high last dose showed that the protection against genetic damage increased with number of repetitions, reaching a maximum at 5-20 times, reflected as low muta-

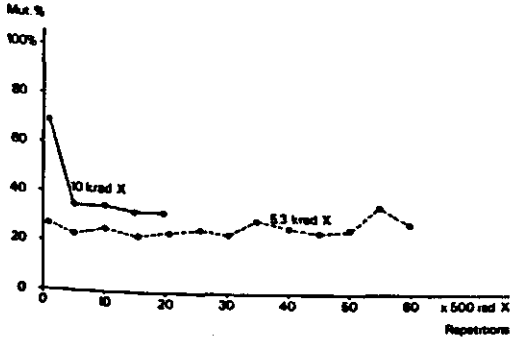


Fig. 29. Effect on mutation frequency of repeated X irradiation with 500 rad at 8-h intervals. Second dose was 10 krad and 5.3 krad (= average of all second doses). Dose rate was 200 rad/min.

tion frequencies. Lower last doses did not respond to number of repetitions, as is demonstrated by the 5.3 krad X line, being the average of all last doses applied. Fast neutrons responded exactly in the same way.

Since survival as well as production clearly reacted upon increasing number of repetitions (figs. 20, 21, 22 and 23), it is *a priori* to be expected that the number of mutants per 100 leaves will increase with increasing number of repetitions. This can be seen in figs. 30 and 31, which demonstrate an optimum number of mutants per 100 leaves around 25-30 repetitions. With X-rays, the highest number was obtained after 30 repetitions, separated by 8-h intervals, and applying a last dose of 4.5 krad X-rays, namely 435 mutants per 100 leaves. This is 130% in comparison with the corresponding optimum acute dose of 3 krad (Table 9). With fast neutrons similar results were obtained (Fig. 31): the highest number, which happened to be the same as with X-rays, namely 435, was obtained after 25 repetitions with 170 rad and a last dose of 1.7 krad. In comparison with the corresponding optimum acute dose of 1.5 krad the highest number amounted to 182% (see Table 9).

The "170 rad Nf line" of Fig. 31 seems to be an indication that the gradually decreasing production (Fig. 23) with increasing number of repetitions is compensated by an increasing mutation frequency, resulting in a more or less steady number of mutants per 100 leaves of that line (the survival stays 100% throughout the whole treatment: Fig. 22).

By comparing Fig. 30 with Fig. 31 it is obvious that fast neutrons, despite their similar action to X-rays upon the material, do have a somewhat more direct effect, demonstrated by the almost horizontal lines of Fig. 30

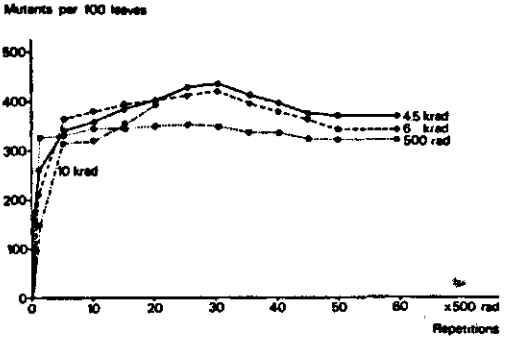


Fig. 30. Effect on number of mutants per 100 leaves of repeated X-irradiation with 500 rad at 8-h intervals; various last doses were applied. Dose rate was 200 rad/min.

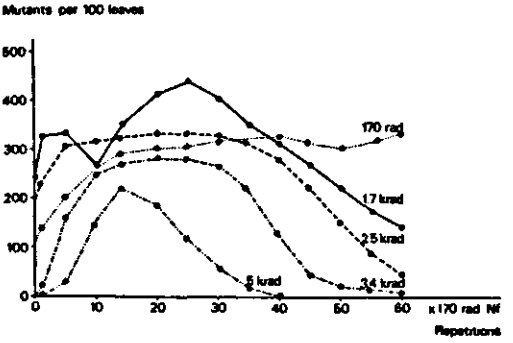


Fig. 31. Effect on number of mutants per 100 leaves of repeated fast neutron irradiation with 170 rad at 8-h intervals; various last doses were applied. Dose rate was 17 rad/min.

Table 9. Number of mutants per 100 leaves (n) and percentage of dwarfs after various X-ray or fast neutron treatments[1].

Treatment	n of the treatment (n_t)	n of the corresponding optimum acute dose (n_o)	n_t/n_o (%)	dwarfs (%)
X-rays				
ACUTE (200 rad/min)				
3 krad (= optimum acute dose)	330	330	100	55
CHRONIC				
5.3 rad/min; 10.9 krad	275	220	125	58
7.3 rad/min; 13.2 krad	255	220	116	53
FRACTIONATED				
Initial dose				
500 rad-8 h-6 krad	415	255	163	57
Repeated				
30 x 500 rad; 4.5 krad	435	335	130	59
Fast neutrons				
ACUTE (17 rad/min)				
1.5 krad (= optimum acute dose)	270	270	100	61
CHRONIC				
0.17 rad/min; 6.4 krad	333	225	148	45
8 rad/min; 1.2 krad	317	225	141	54
57 rad/min; 1 krad	335	225	149	81
FRACTIONATED				
Initial dose				
250 rad-8 h-1.7 krad	505	345	146	52
Interval				
170 rad-24 h-3.4 krad	355	250	142	58
Repeated				
25 x 170 rad; 1.7 krad	435	238	182	56

1. Compilation of data from various experiments.

(after approx. 20 repetitions with 500 rad X-rays) and the rapidly decreasing lines of Fig. 31, after reaching the optimum. But here, again, we have to remember that the fast neutron repeated irradiations were carried out with the sub-optimum dose of 170 rad.

The foregoing was a description of the experimental results which, though interesting, do not elucidate much of the origin of the phenomena. What actually takes place in plants is not known, since no or very scanty and incomplete investigations in other angiosperms have been reported. The comparison with the more extensive and more complete radiobiological investigations in bacteria and

animals or animal (mammalian) tissues is risky and probably not justified, as has been discussed before (Section 3.3.1.5).

3.3.3 Practical implications

Although a clear-cut protective effect of fractionated irradiation is demonstrated, the practical results, measured as number of mutants per 100 leaves in percentage of the corresponding optimum acute dose, are somewhat disappointing. The number of mutants can be increased, in the case of X-rays, to 130% by applying 30 repetitions of 500 rad X-rays, separated by 8-h intervals and a last dose of 4.5 krad and to 163% by using an initial dose of 500 rad, 8 hours later followed by 6 krad. However, this high figure is somewhat questionable when compared with a similar treatment, which resulted in only 90% (see discussion in Section 3.3.2 under *time interval*). More important is that the mutation spectrum, expressed as percentage of dwarfs (last column, Table 9), turned out to be unchanged in comparison to acute treatments.

The same holds true for fast neutrons which, compared with the optimum acute dose of 1.5 krad at 17 rad/min, resulted in 182% (25 repetitions of 170 rad, followed by 1.7 krad), 142% (170 rad - 24 hours - 3.4 krad) and 146% (250 rad - 8 hours - 1.7 krad). However, when these figures are expressed in the optimum 1 krad acute dose administered with a higher dose rate (57 rad/min), giving 149% as compared to the optimum 1.5 krad acute dose at 17 rad/min, the situation becomes different. Then only the repeated irradiation shows a surplus in number of mutants per 100 leaves, namely 122% (182/149 x 100). The mutation spectrum, if the % of dwarfs is taken as a standard for it (discussed in Section 3.1.2), is unchanged (last column, Table 9) and completely comparable with that after X-rays (Table 7, page 32).

However, the missing links in these considerations are fractionated irradiations with the optimum initial dose of 500 rad fast neutrons. A single pre-treatment or a repeated irradiation with that dose might have rendered an equally larger number of mutants per 100 leaves (even when expressed in the absolute optimum acute dose of 1 krad at 57 rad/min) as has now been found, using 170 rad as initial dose (and compared to 1.5 krad at 17 rad/min). In other words, it is not justified to base a decision about the practical value of a given fast neutron treatment at a dose rate of 17 rad/min on the comparison with the absolute optimum acute dose of 1 krad at 57 rad/min.

Therefore it could be worthwhile to consider a pre-treatment, with the optimum initial X-ray dose, before a heavy second dose. A repeated irradia-

tion, in the case of fast neutrons, seems promising, but since it is too costly and time consuming, it has to be rejected as impractical.

4 Comparison of acute, chronic and fractionated irradiations in relation to (commercial) plant breeding

As has been discussed in the introduction (Section 1.1), one of the starting-points of my investigations was the expectation that repair of physiological and genetical effects might be the result of partly different processes. Consequently it was anticipated that, by controlling the various factors of influence on these processes, it would be possible to improve the efficiency of the mutagenic treatment in such a way, that more and possibly also more useful mutants were obtained from a given number of irradiated leaves.

The general impression from the data presented in the foregoing chapters is, that this expectation has not been realized. The difference between acute and chronic irradiations (Table 9) are slight and not interesting in the case of X-rays, and larger, but sometimes impractical, in the case of fast neutrons (6.4 krad at a dose rate of 0.17 rad/min requires approx. 26 full days irradiation!; 1.2 krad at a dose rate of 8 rad/min, however, is applicable since it requires only 2.5 hours).

The previous statement at first appears to be in contrast with most of the curves, which show an optimum value for the parameter studied. However, on further consideration, the optima in figs. 25, 26, 28, 30 and 31, which represent the number of mutants per 100 leaves, are the result of the multiplication of two physiological parameters (survival and production) and one genetic parameter (mutation frequency). This can be seen in the last but one column of Table 5 (page 28), combined with the 4th column: the number of mutants per 100 leaves is A x B x D x 15.10^{-4}, in which A represents survival, B represents production and D represents mutation frequency.

The reaction of either survival and production, which show a high degree of correlation with each other, consists of a generally drastic increasing percentage after the gradual increase of certain factors, such as initial dose, time interval and number of repetitions. Reversely, mutation frequency shows a decreasing and less pronounced percentage, following the gradual increase of the factors mentioned before. Consequently, an optimum is the result, when the three parameters are combined, as is the case when the result of a given treatment is expressed as the number of mutants per 100 irradiated leaves.

Because of the difference in intensity upon changing factors, such an optimum would also be obtained when only one physiological parameter (or the square root of the product of survival and production) is multiplied by the mutation frequency. Moreover, one gets the impression that the mutation frequency does not only react less intensively upon varying factors but also slower, when compared with survival and production. In Fig. 24 for instance the minimum and maximum mutation frequency, after various initial doses, varies from approx. 20% to approx. 60-80% for fast neutrons (the high figures for mutation frequency are not significant) whereas it varies from approx. 35% to 70% for X-rays. This is much less in comparison with the magnitude observed when survival and production are used as parameter (figs. 12 and 13: survival ranges from 20-100% (X-rays) and from 0-approx. 70% (fast neutrons), whereas production ranges from approx. 10-80% (X-rays) and 0-60% (fast neutrons)).

In the same Fig. 24 the minimum mutation frequency lies around an initial dose of 0.8 krad for fast neutrons and 1.5 krad for X-rays (the minima after an initial dose of approx. 150 rad have been neglected: see Section 3.3.2). This is in contrast to the optimum initial dose of 500 rad for both X-rays and fast neutrons in the case of survival and production (figs. 12 and 13). This indicates, that maximum protection against genetic damage is obtained after a higher initial dose as compared with the protection against damage of a more physiological nature.

This causes the optimum which is obtained by the multiplication of survival, production and mutation frequency to be somewhat diffuse and to vary with variations in treatment and circumstances. But the fact remains, that the adventitious bud technique, in which survival, production and mutation frequency are logical and inseparable factors, does result in maximum number of mutants per given number of leaves following a certain treatment.

For commercial mutation breeding purposes a few questions arise, namely:
1. which treatment is to be preferred taking into consideration the number and quality of the mutants,
2. which treatment should be selected, taking into account the availability of leaves in connection with the availability of greenhouse space (with other words: a very heavy treatment, requiring many leaves and less greenhouse space to produce a given number of mutants or a light treatment, requiring less leaves but more space to obtain a similar number of mutants, based on the assumption that the % of dwarfs is a measure for the quality of the mutation spectrum), and
3. which irradiation facility is to be preferred, taking into account the

availability as well as economical aspects.

As is shown in Table 9, page 57, and as has been discussed in various Chapters before, the only practical treatment with X-rays is either an acute dose of 3 krad or a heavy dose of approx. 6 krad preceeded by an initial dose of 500 rad and an 8-h interval between first and second dose. The mutation spectrum seems to be alike in all cases.

Using fast neutrons, a semi-acute irradiation with 8 rad/min and a total dose of 1.2 krad seems to give the best results; an acute irradiation with 57 rad/min unfortunately is accompanied by an unfavourable mutation spectrum (81% dwarfs, last column Table 9). Two other treatments give similar results (the fractionated treatments in Table 9) and might also be considered. All other treatments, however, are impractical or too costly (repeated irradiation, for instance).

The choice between a light or heavy dose depends on the availability of leaves to be irradiated, and greenhouse space. Since the latter is usually the limiting factor a heavy dose seems to be the best choice. When both are not limited, a light dose is to be preferred, mainly with respect to a possible disastrous effect of unforeseen unfavourable (climate) conditions as well as the possibility of an unfavourable effect upon the mutation spectrum, both discussed before. Moreover, the chance that a favourable mutation is accompanied by one or more unfavourable ones, is reduced.

5 Comparison of X-rays and fast neutrons

The qualitative difference in the underlying work between X-rays and fast neutrons has been shown to be extremely small, although the reactions of the material after neutrons were not as clear-cut as with X-rays, which point towards a more direct effect of fast neutrons. The difference between both radiation types is reflected by the fact that neither the dose rate, nor dose fractionation has an effect upon the mutation spectrum when X-rays are used; fast neutrons, however, show an unfavourable shift of the spectrum when high dose rates are applied (Table 8, page 37).

When, however, the effects of X-rays and fast neutrons (upon survival, production and mutation frequency) are compared on a rad basis, fast neutrons are sometimes more efficient, which can be expressed in terms of an RBE (relative biological effectiveness). For acute irradiations, using 17 rad/min, fast neutrons have an RBE = 2, compared with X-rays (figs. 1, 2 and 3). When a higher fast neutron dose rate is applied, namely 57 rad/min, the RBE value is 3 (Fig. 9). In fractionation experiments a more complicated picture was observed. For the induction of a protective effect the RBE = 1, when survival and production are used as parameters (Fig. 6). When the number of mutants is the end-point, the RBE = 2 for initial dose, and 1 for number of repetitions.

A striking equality between the two radiation types is shown in figs. 17 and 18 where it is shown that it does not make much difference, upon survival and production, whether X-rays or fast neutrons are used, either as initial dose or as second dose. This is an unexpected result, since the completely different way of energy dissipation in (biological) material of X-rays (sparsely ionizing) and fast neutrons (densely ionizing mainly through recoil protons) *a priori* presupposes a more pronounced difference in effect. The only explanation is, that despite the different ways of energy dissipation, the changes in the end-points measured are the result of a series of indirect reactions, which largely follow the same pathways.

Consequently it is hard to say which of the two radiation-types is to be preferred in our material. The heterozygosity of the *Saintpaulia* cultivar used is apparently so large, that an increase in mutation frequency (by using

various treatments or by using neutrons) is limited, probably because cells, carrying too many genetic changes disappear. This may explain the almost constant percentage dwarf mutants (Table 9) in spite of the different treatments and the different radiation methods used. (The only exception is the high % of dwarfs after a fast neutron irradiation with 57 rad/min (Table 8, page 37 and Table 9, page 57).

In species, however, which have a much lower degree of heterozygosity, it might pay to investigate whether or not fast neutrons (with a high dose rate) are superior to X-rays.

Summary

Detached leaves of African Violet, *Saintpaulia ionantha* H. Wendl. cv. Utrecht, have been exposed to acute, chronic or fractionated irradiations with X-rays or fast neutrons to define the optimum treatment for commercial mutation breeding. The effects of the various treatments were expressed as:

1. survival of the irradiated leaves,
2. production of adventitious plantlets at the base of the petiole and
3. mutation frequency and number of mutants per 100 leaves.

The plantlets formed on the petiole of *Saintpaulia* leaves have two characteristics which make them especially useful for such a study. First, one can be certain that throughout the time of irradiation and treatment all cells likely to be involved in the production of adventitious plantlets are in a non-dividing state. Second, the apex of each adventitious plantlet can be traced back ultimately to cells derived from a single epidermal cell. The evidence for this is that all mutants (with a few exceptions discussed in this report) are complete: sectors are virtually never found.

The results show a striking dose rate effect on survival, production and mutation frequency. At a rate of 2 rad/min the LD_{50} for survival is obtained after approx. 100 krad X-rays as compared to approx. 5 krad X-rays at a rate of 200 rad/min. The critical dose rate, at which there is a maximum effect from a change in dose rate proved to be approx. 7 rad/min (X-rays).

Dose fractionation experiments (experiments in which more than one irradiation is made, with the irradiations separated by a time interval) have demonstrated, that a relatively low initial dose induces a mechanism which protects the leaves against part of the effect of one or more subsequent radiation doses. The optimum initial dose for maximum protection was found to be 500 rad of either X-rays or fast neutrons. The optimum interval, after which the protection has reached a maximum, was 8-12 hours, depending on the size of the initial dose and also on the size of the subsequent dose(s). The extent of the protection was shown to be equivalent to 3-4 krad X-rays or 1.5-2 krad fast neutrons, after a single pre-treatment with the optimum initial dose. The protection was approx. double that amount after 10-15 repeated treatments with

the optimum initial dose, separated by 8-h intervals.

There is another way of considering the magnitude of the protection afforded by the initial dose. Normally a dose of 6.5 krad of X-rays is lethal. After one initial treatment with 500 rad (of X-rays) one gets approx. 100% survival (see the 6 krad X line in Fig. 12), whereas 25% survival is obtained with a second dose as high as 10 krad. After 5 repeated irradiations with the same initial dose, separated by 8-h intervals, 100% survival is obtained after a last dose of 10 krad.

Generally, survival and production as well as number of mutants reacted similarly upon different types of treatments. Since the first two parameters, however, reacted much more intensively upon variation of various factors, compared with the third parameter, mutation frequency, it was possible to calculate and define optimum treatments.

On the basis of number of mutants per 100 irradiated leaves, as a percentage of the corresponding optimum acute dose (3 krad X-rays or 1.5 krad fast neutrons) it was shown that a higher number of mutants can be obtained with certain dose rate treatments or fractionated irradiations compared with an optimum acute irradiation.

Examples are a pre-treatment with 500 rad and a second dose of 6 krad, separated by an 8-h interval in the case of X-rays or a similar treatment with fast neutrons (Table 9). Other treatments are impractical or too expensive because of prolonged irradiations.

The difference between X-rays and fast neutrons (average energy of approx. 1.7 MeV) has shown to be surprisingly small and one can hardly find a reason to prefer one type of radiation to the other.

The results described in this report are valid only for *Saintpaulia*, however. In other crops, which might be less heterozygous and thus produce fewer mutants, an increase in number of mutants or a shift in the mutation spectrum may be more significant.

The significance of the adventitious bud technique for mutation breeding in general has been discussed especially with regard to the role of diplontic selection. Reasons are presented to support the belief that there is much less diplontic selection with the adventitious bud technique than in normal multicellular apices.

Acknowledgements

Leaves of African Violet were first irradiated late in 1960 and have continued since on an ever increasing scale. Many persons assisted, so that it is hard to recollect everybody's true contribution. Nevertheless I express my sincere gratitude to:

Seymour Shapiro, for introducing me to the field of mutagenesis and radiobiology and for his continued interest.

Dick de Zeeuw, for his confidence in my work and, above all, in giving me so much freedom of action.

Professor Sneep, for the many inspiring and constructive discussions.

Many of my colleagues, who patiently listened for years to *Saintpaulia* stories over coffee, lunch and tea-breaks and on many other occasions and very many other ITAL staff members: my technicians Anneke, Heleen, Marian and Emmy; the greenhouse attendants Leo, Adri, Wim and Hans; all the reactor staff, who at times worked night and day for long periods on some complicated (fast) neutron or X-ray experiments; the typists and during the final stage Hans in particular, who typed and retyped texts; Jan for the drawings; Menno for the many fine pictures; and many others, who in one way or another have contributed to the final result.

Pudoc, for helping and advising with form, content, style and English.

And last but not least my wife and children, who understandingly accepted my sometimes poor behaviour as husband and father during the times I was thinking and dreaming of her majesty *Saintpaulia* and her adventitious buds.

References

Ainsworth, E.J. & G.F. Leong, 1966. Recovery from radiation injury in dogs as evaluated by the split-dose technique. Radiat. Res. 29: 131-142.

Amato, F. d', 1972. Morphogenetic aspects of the development of meristems in seed embryos. In: Dynamics of meristem cell populations; Plenum Press, N. York (in press).

Ancel, S., 1928. L'effet du fractionnement des doses de rayons X sur des graines germinées. C.r. Séanc. Soc. Biol. 98: 223-224.

Anonymus, 1963. Irradiation facilities of the Association Euratom-ITAL 1: X-ray machine.

Anonymus, 1967. Neutron irradiation of seeds. IAEA techn. Rep. 76.

Anonymus, 1968. Dose rate in mammalian radiation biology. U.S.A. EC-DTI Conf.

Anonymus, 1970. Manual on mutation breeding. FAO/IAEA techn. Rep. Ser. 119. Vienna.

Bacchetti, S., 1965. Recovery from sublethal X-ray damage in surviving yeast cells. Radiat. Res. 25: 103-114.

Bacchetti, S., R. Elli, R. Falchetti & F. Mauro, 1965. Recovery from X-ray damage in yeast cells. Ann. Meeting Eur. Soc. Radiat. Biol., Utrecht, The Netherlands.

Bacq, Z.M. & P. Alexander, 1961. Fundamentals of radiobiology. Pergamon Press, Oxford, London, New York, Paris.

Balkema, G.H., 1971. Chimerism and diplontic selection. A.A. Balkema, Rotterdam-Kaapstad.

Bergann, F., 1966. The relative instability of chimerical clones - the basis for further breeding. Gatersleben Symp. on induced mutations and their utilization: 287-301.

Bergann, F., 1967. Mutations-Chimären: Rohmaterial züchterischer Weiterbehandlung. Umschau 67: 791-797.

Bewley, D.K., J.F. Fowler, R.L. Morgan, J.A. Silvester, B.A. Turner & R.H. Thomlinson, 1963. Experiments on the skin of pigs with fast neutrons and 8 MeV X-rays, including some effects of dose fractionation. Br. J. Radiol. 36: 107-115.

Bewley, D.K., S.B. Field, R.L. Morgan, B.C. Page & C.J. Parnell, 1967. The response of pigskin to fractionated treatments with fast neutrons and X-rays. Br. J. Radiol. 40: 765-770.

Bhojwani, S.S. & B.M. Johri, 1970. Cytokinin-induced shoot bud differentiation in mature endosperm of *Scurrula pulverulenta*. Z. Pfl. Physiol. 63: 269-275.

Bottino, P.J. & A.H. Sparrow, 1970. The effects of exposure time and rate on the survival and yield of lettuce, barley and wheat. In: (Bensen & Sparrow, ed.); Survival of food crops and livestock in the event of nuclear war: 306-324. Upton, N.Y.

Bowen, H.J.M., 1965. Mutations in horticultural chrysanthemums. In: The use of induced mutations in plant breeding. Rep. FAO/IAEA techn. Meeting Rome, 1964: 695-700. Pergamon Press, Oxford.

Braun, H., 1957. Experimentelle Untersuchungen über die Wirkung von Röntgenganzbestrahlungen auf den tierischen Organismus bei nachfolgender Bestrah-

lung mit Letaldosen. Oncologia 10: 272.
Braun, H., 1962. Über die Steigerung der Leistungsfähigkeit der Ratten nach Ganzkörperbestrahlung, geprüft im Schwimmversuch. Strahlungstherapie 119: 462.
Briggs, R.W., 1968. Effects of post-treatment hydration and dehydration of ethyl methane sulphonate-treated maize seeds. In: Mutations in plant breeding 2: 47-57. IAEA, Vienna.
Broerse, J.J., 1966. Effects of energy dissipation by mono-energetic neutrons in mammalian cells and tissues. Radiobiologisch Instituut TNO, Rijswijk.
Broertjes, C., 1965. The organization and co-ordination of mutation breeding research in the Netherlands. Meded. LandbHogesch. Gent 30: 907-975.
Broertjes, C., 1968a. Mutatieveredeling bij land- en tuinbouwgewassen. Landbouwk. Tijdschr. 80: 162-168.
Broertjes, C., 1968b. Dose-rate effects in *Saintpaulia*. In: Mutations in plant breeding 2: 63-71. IAEA, Vienna.
Broertjes, C., 1969. Mutation breeding of *Streptocarpus*. Euphytica 18: 333-339.
Broertjes, C., 1970. Dose fractionation studies and radiation-induced protection phenomena in African Violet. In: (Bensen & Sparrow, ed.); Survival of food crops and livestock in the event of nuclear war: 325-342. Upton, N.Y.
Broertjes, C., 1971. Possibilities of mutation breeding in ornamentals. In: Eucarpia meeting on ornamentals: 7-15, Wageningen.
Broertjes, C., 1972a. Improvement of vegetatively propagated plants by ionizing radiation. In: Induced mutations and plant improvement: 293-299. IAEA, Vienna.
Broertjes, C., 1972b. Mutation breeding of *Achimenes*. Euphytica 21: 48-63.
Broertjes, C. & H.Y. Alkema, 1970. Mutation breeding of flowerbulbs. In: First int. Symp. on flowerbulbs 2: 407-411, Noordwijk/Lisse.
Broertjes, C., B. Haccius & S. Weidlich, 1968. Adventitious bud formation on isolated leaves and its significance for mutation breeding. Euphytica 17: 321-344.
Broertjes, C. & L. Leffring, 1972. Mutation breeding of *Kalanchoë*. Euphytica (in press).
Bryant, P.E., 1968. Survival after fractionated doses of radiation: modification by anoxia of the response of *Chlamydomonas*. Nature, Lond. 219: 75-77.
Buvat, R., 1952. Structure, évolution et fonctionnement du méristème apical de quelques dicotylédones. Annls. Sci. nat. (Bot.), 11e sér. 13: 202-203.
Casarett, A.P., 1968. Radiation biology. Prentice-Hall, N.Yersey. US AEC.
Chaput, R.L. & R.T. Kovacic, 1970. Miniature pig performance after fractionated supralethal doses of ionizing radiation. Radiat. Res. 44: 807-820.
Chadwick, K.H. & H.P. Leenhouts, 1972. The molecular target theory and the analysis of survival curves (in preparation).
Chadwick, K.H. & W.F. Oosterheert, 1969. Neutron spectrometry and dosimetry in the subcore facility of a swimming pool reactor. Atompraxis 15: 178-180.
Christian, E.J.B., S.Ph. Stearner & M.H. Sanderson, 1965. Mechanisms of γ-ray induced radio-resistance to early mortality in the 3-day chick. Radiat. Res. 25: 179.
Clowes, F.A.L., 1965. Synchronization in a meristem by 5-amino-uracil. J. exp. Bot. 16: 581-586.
Constantin, M.J., D.D. Killion & E.G. Siemer, 1970. Exposure rate effects on soybean plant responses to gamma radiation. In: (Bensen & Sparrow, ed.); Survival of food crops and livestock in the event of nuclear war: 277-286. Upton, N.Y.
Cronkite, E.P., C.R. Sipe, O.C. Eltzholtz, W.H. Chapman & F.W. Chambers Jr., 1950. Increased tolerance of mice to lethal X-irradiation as a result of previous sub-lethal exposures. Proc. Soc. Exp. Biol. Med. 73: 184-186.
Dacquisto, M.P., 1959. Acquired radioresistance. A review of the literature and report of a confirmatory experiment. Radiat. Res. 10: 118-129.

Davies, D.R., 1962. The effect of dose-fractionation on mutation induction. Strahlenwirkung und Milieu: 160-170.
Davies, D.R. & E.T. Wall, 1960. Induced mutations at the V^{by} locus of *Trifolium repens*. I. Effects of acute, chronic and fractionated doses of gamma radiation on induction of somatic mutations. Heredity 15: 1-15.
Denekamp, J., J.F. Fowler, K. Kragt, C.J. Parnell & S.B. Field, 1966. Recovery and repopulation in mouse skin after irradiation with cyclotron neutrons as compared with 250-keV X-rays or 15-MeV electrons. Radiat. Res. 29: 71-84.
Dertinger, H. & H. Jung, 1969. Molekulare Strahlenbiologie. Springer, Verlag Berlin-Heidelberg.
Dimitrov, A., 1964. Untersuchungen an Tieren mit "Immunität" gegen ionisierenden Strahlen. Strahlentherapie 124: 114.
Dommergues, P., 1962. La destinée de la cellule mutée: conséquences dans le cas des plantes à multiplication végétative et dans le cas des plantes à reproduction sexuée. Proc. 3rd Eucarpia Congr., Paris: 115-139.
Dommergues, P. & J. Gillot, 1964. Variation de réaction des boutures d'oeillet à l'irradiation gamma. In: The use of induced mutations in plant breeding, Rome: 713-718.
Ebert, M. & A. Howard, 1963. Radiation effects in physics, chemistry and biology. North-Holland Publishing Co., Amsterdam.
Ehrenberg, L. & N. Nybom, 1954. Ion density and biological effectiveness of radiations. Acta agric. scand. 4: 396-418.
Elkind, M.M., 1970. Damage and repair processes relative to neutron (and charged particle) irradiation. Curr. Topics Radiat. Res. Q. 7: 1-44.
Emery, E.W., J. Denekamp & M.M. Ball, 1970. Survival of mouse skin epithelial cells following single and divided doses of X-rays. Radiat. Res. 41: 450-466.
Evans, H.J., 1966. Repair and recovery from chromosome damage after fractionated X-ray dosage. In: Genetical aspects of radiosensitivity: mechanisms of repair: 31-48. IAEA, Vienna.
Evans, H.J., 1967a. Repair and recovery from chromosome damage induced by fractionated X-ray exposures. Radiat. Res. (Proc. 3rd internat. congr. of radiat. research; Cortina d'Ampezzo, 1966). Ed. G. Silini; North-Holland Publ. Co. - Amsterdam: 482-501.
Evans, H.J., 1967b. Repair and recovery at chromosome and cellular levels: similarities and differences. In: Recovery and repair mechanisms in radiobiology. Brookhaven Symposia in Biology, Number 20: 111-131.
Fujii, Y., 1968. Fractionation experiments with γ-rays and neutrons in mais. Ann. Rep. natn. Inst. Genet. Jap. 19: 78-79.
Gaul, H., 1957. Zur Frage der ontogenetischen Elimination mutierter Zellen nach Röntgenbestrahlung von Samen. Naturwissenschaften 44: 566.
Gaul, H., 1963. Mutationen in der Pflanzenzüchtung. Z. PflZücht. 50: 194-307.
Gaul, H., K. Bender, E. Ulonska & M. Sato, 1966. EMS-induced genetic variability in barley; the problem of EMS-induced sterility, and a method to increase the efficiency of EMS-treatment. In: Mutations in plant breeding: 63-85. IAEA, Vienna.
Generalova, M.V., 1968. The effect of the X-ray fractionated dose on the chromosome aberrations yield in *Crepis capillaris*. I. Irradiation at the G_1-stage. Genetika 4: 17-23.
Generalova, M.V., 1969. The effect of fractionated X-ray doses on the yield of chromosome aberrations in *Crepis capillaris*. II. Irradiation at the stages G_1, S and G_2. Genetika 5: 84-92.
Graevskaja, B.M. & R.J. Kejlina, 1956. Die Herabsetzung der Empfindlichkeit von Tieren gegen Einwirkung von Röntgenstrahlen in letaler Dosis bei ihrer vorhergehenden Bestrahlung mit nicht letalen Dosen. Biofizika 1: 230.

Heide, O.M., 1965. Interaction of temperature, auxins and kinins in the regeneration ability of begonia leaf-cuttings. Physiologia Pl. 18: 891-920.
Heslot, H., 1964. L'induction expérimentale de mutations chez les plantes florales. C.r. Acad. Agric. Fr., Séance du 16 Décembre: 1281-1308.
Hoffman, N.A., F.F. Gollin & K.H. Clifton, 1965. Gross tumor response versus quantitative cell survival following irradiation; comparison and interpretation. Radiat. Res. 25: 199.
Holloway, R.J., G.F. Leong, E.J. Ainsworth, M.L. Albright & S.J. Baum, 1968. Recovery from radiation injury in the hamster as evaluated by the split-dose technique. Radiat. Res. 33: 37-49.
Horsley, R.J. & A. Laszlo, 1971. Unexpected additional recovery following a first X-ray dose to a synchronous cell culture. Int. J. Radiat. Biol. 20: 593-596.
Howard, A., 1968. The oxygen requirement for recovery in split-dose experiments with *Oedogonium*. Int. J. Radiat. Biol. 14: 341-350.
Kabakova, N.M., Yu.G. Kapul'tsevitch & V.I. Korogodin, 1970. Influence du fractionnement des doses d'irradiation sur la survie des cellules de levures (en Russe). Radiobiologyia 10: 300-303.
Kada, T., C.O. Doudney & F.L. Haas, 1966. Mutation frequency response to a second radiation exposure. Mutation Res. 3: 118-128.
Kampen, E.J. van, 1959. Ioniserende straling. Chem. Weekbl. 55: 153-158, 165-170.
Kaplan, R.W., 1951. Chromosomen- und Faktormutationsraten in Gerstenkörnern bei verschiedenartigen Quellungsbehandlungen oder Kälte während oder nach der Röntgenbestrahlung sowie bei Dosisfraktionierung. Z. indukt. Abstamm.- u. VererbLehre 83: 347-382.
Kaplan, H.S., 1971. Chemical inhibitors of the repair of X-ray induced single strand breaks in cellular DNA. Rep. 8th ann. Meet. Eur. Soc. Radiat. Res., Basko Polje, Yugoslavia.
Kiefer, J., 1966. X-ray effects on barley roots. II. Single and fractionated exposure. Int. J. Radiat. Biol. 11: 373-382.
Klöpfer, K., 1966. Methods of demonstrating and breeding chimerical potato clones. Gatersleben Symp. on induced mutations and their utilization: 305-309.
Krokowski, E., 1968. Extraregionale Strahlenwirkungen. Strahlentherapie 135: 193-201.
Krokowski, E. & V. Taenzer, 1966. Der radiogene Strahlenschutzeffekt. Strahlentherapie 130: 139-145.
Lane, G.R., 1952. Interpretation in X-ray chromosome breakage experiments. Genetics 37, suppl.: 23-34.
Lawrence, C.W., 1968. Effect of fractionated doses of radiation on recombination. Heredity 23: 143-146.
Leidl, W. & H. Zankl, 1970. Studies about the effect of interval length in fractionated irradiation of rabbit testes. Strahlentherapie 139: 548-552.
Leonard, A. & Gh. Deknudt, 1971. The rate of translocations induced in spermatogonia of mice by two X-irradiation exposures separated by varying time intervals. Radiat. Res. 45: 72-79.
Littlebrand, B. & L. Révész, 1967. Effect of a second exposure to X-rays on post-exposure recovery. Nature, Lond. 214: 841-842.
Maisin, J., E. van Duyse, A. Dunjic, J. van der Merckt, A. Wambersie & D. Werbrouck, 1960. Acquired radio-resistance, radio-selection and radio-adaptation. Int. J. Radiat. Bot., suppl. 183-194.
Menhenett, R., 1970. Effects of adenine on the formation of roots and buds in leaf squares. New Phytol. 69: 537-547.
Micke, A., 1970. Factors influencing the mutant spectrum and the quality of mutants. Type of mutagen and dose. In: Manual on mutation breeding. IAEA

techn. Rep. 119: 125-129.
Miller, M.W. & J.S. Colaiace, 1970. Radiation dose fractionation, mitotic index and multiple fixation studies on chromosome aberration in *Vicia faba*. Mutation Res. 10: 449-462.
Moore Jr., H.E., 1957. African violets, gloxinias and their relatives. A guide to the cultivated gesneriads. MacMillan, New York.
Morris, T. & S.E. O'Grady, 1970. Dose-response curve for X-ray induced translocations in mouse spermatogonia. II. Fractionated doses. Mutation Res. 9: 411-415.
Nachtwey, D.S., E.J. Ainsworth & G.F. Leong, 1967. Recovery from radiation injury in swine as evaluated by the split-dose technique. Radiat. Res. 31: 353-367.
Nag, K.K. & B.M. Johri, 1970. Effects of cytokinins and injury on the formation of shoots by leaves of *Dendrophthoe falcata*. Planta 90: 360-364.
Narayanan, K.L. & C.F. Konzak, 1969. Influence of chemical post-treatments on the mutagenic efficiency of alkylating agents. In: FAO/IAEA, Induced mutations in plants: 281-304. Pullman, Wash.
Naylor, E. & B. Johnson, 1937. A histological study of vegetative reproduction in *Saintpaulia ionantha*. Am. J. Bot. 24: 673-678.
Neary, G.J., 1969. The interpretation of survival curves in relation to radiation quality and cellular repair mechanisms. Proc. 2nd Symp. Microdosimetry, Stresa, Italy: 51-74.
Nettancourt, D. de, P. Dijkhuis, A.J.G. van Gastel & C. Broertjes, 1971. The combined use of leaf irradiation and of the adventitious bud technique for inducing and detecting polyploidy, marker mutations and self-compatibility in clonal populations of *Nicotiana alata* Link and Otto. Euphytica 20: 508-521.
Nilan, R.A., 1966. Nature of induced mutations in higher plants. Gatersleben Symp. on induced mutations and their utilization: 5-20.
Nybom, N., 1961. The use of induced mutations for the improvement of vegetatively propagated plants. In: Mutations and plant breeding: 252-294. National Academy of Sciences, Washington.
Nybom, N. & A. Koch, 1965. Induced mutations and breeding methods in vegetatively propagated plants. In: The use of induced mutations in plant breeding. Rep. FAO/IAEA techn. Meeting, Rome, 1964: 661-678. Pergamon Press, Oxford.
Page, N.P., D.S. Nachtwey, G.F. Leong, E.J. Ainsworth & E.L. Alpen, 1965. Recovery from radiation injury in sheep, swine and dogs as evaluated by the split-dose technique. Radiat. Res. 25: 54 (abstract).
Paribok, V.P. & G.F. Krupnova, 1970. The nature of the effect of fractionated X-irradiation of meristematic cells. Citologya 12: 525-533.
Paterson, E., C.W. Gilbert & M. Haigh, 1956. Effects of paired doses of whole body irradiation in the rhesus monkey. Br. J. Radiol. 29: 218.
Paterson, E. & J. Matthews, 1952. Time intensity factors and whole body irradiation. Br. J. Radiol. 25: 427.
Platzman, R.L., 1958. Radiation biology and medicine. Reading, Addison-Wesley Press.
Plummer, T.H. & A.C. Leopold, 1970. Chemical treatment for bud formation in *Saintpaulia*. Proc. Am. Soc. hort. Sci. 70: 442-444.
Pötsch, J., 1966. On the dissociation of chimerical shoot variants by the use of X-rays. Gatersleben Symp. on induced mutations and their utilization: 301-304.
Prensky, W. & H.H. Smith, 1965. The mechanism of 5-amino-uracil induced synchrony of cell division in *Vicia faba* root meristems. J. Cell Biol. 24: 401-414.
Puite, K.J., D.L.J.M. Crebolder, J.J. Broerse & B. Hogeweg, 1972. Inter-

comparisons of absorbed dose and absorbed dose distribution for X-irradiations using mailed LiF thermoluminescent dosimeters. Phys. Med. Biol. (in press).
Ramsell, T.G. & R.J. Berry, 1966. Recovery from X-ray damage to the lens. The effects of fractionated doses observed in rabbit lens epithelium irradiated in vivo. Br. J. Radiol. 39: 853-858.
Sacher, G.A. & D. Grahn, 1957. Response of mice to conditioning doses of whole body irradiation. Rep. Argonne natn. Lab. biol. med. biophys. Div. 5841: 95-97.
Satina, S. & A.G. Avery, 1940. Demonstration of the three germ layers in the shoot apex of *Datura* by means of induced polyploidy in periclinal chimeras. Am. J. Bot. 27: 895-905.
Sax, K. & H. Luippold, 1952. The effect of fractional X-ray dosage on the frequency of chromosome aberrations. Heredity 6: 127-133.
Smith, H.H., 1961. Mutagenic specificity and directed mutation. In: Mutations and plant breeding. NAS-NRC 891: 413-436.
Sparrow, A.H., 1962. The role of the cell nucleus in determining radiosensitivity. Brookhaven Lecture Series 17, BNL 766 (T-287).
Sparrow, A.H., 1964. Relationship between chromosome volume and radiation sensitivity in plant cells. In: Symp. on fundamental cancer research and cellular radiation biology. Williams and Wilkins Co., Baltimore: 199-218.
Sparrow, A.H., 1965. Comparisons of the tolerances of higher plant species to acute and chronic exposures of ionizing radiation. Jap. J. Genet. 40, suppl.: 12-37.
Sparrow, A.H., R.C. Sparrow & L.A. Schairer, 1960. The use of X-rays to induce somatic mutations in *Saintpaulia*. Afr. Violet Mag. 13: 32-37.
Sparrow, A.H., R.L. Cuany, J.P. Miksche & L.A. Schairer, 1961. Some factors affecting the responses of plants to acute and chronic radiation exposures. Radiat. Bot. 1: 10-34.
Sparrow, A.H., S.S. Schwemmer & P.J. Bottino, 1970. The effects of external gamma radiation from radioactive fall out on plants with special reference to crop production. In: (Bensen & Sparrow, ed.). Survival of food crops and livestock in the event of nuclear war: 670-711. Upton, N.Y.
Stone, W.S., 1955. Indirect effects of radiation on genetic material. In: Brookhaven Symp. on biology 8: mutation: 171-190.
Swift, H., 1953. Quantitative aspects of nuclear nucleoproteins. Int. Rev. Cytol. 2: 1-76.
Sybenga, J., 1964. Quantitative analysis of radiation-induced sectorial discoloration of the first leaf of *Crotalaria intermedia*. Radiat. Bot. 4: 127-139.
Sybenga, J., 1968. Cytogenetica. A. Oosthoek, Utrecht.
Taenzer, V. & E. Krokowski, 1966. Änderung der Radiosensibilität durch Vorbestrahlung. Strahlentherapie 129: 1.
Taenzer, V. & E. Krokowski, 1968. Acquired radioresistance following whole body irradiation. Acta radiol. ther. phys. biol. 7: 88-96.
Tates, A.D., 1968. The lowering effect of dose-fractionation on recessive lethal frequencies in *Drosophila* spermatocytes. Mutation Res. 5: 109-116.
Taylor Jr., F.G., 1965. Nuclear volume changes in south-eastern tree species before spring growth. Radiat. Bot. 5: 61-64.
Tazima, Y. & K. Onimaru, 1966. Repair of radiation induced permutational damages revealed by fractionated irradiation of silkworm spermatids. Ann. Rep. natn. Inst. Genet. Japan 17: 91-92.
Withers, H.R. & M.M. Elkind, 1969. Radiosensitivity and fractionation response of crypt cells of mouse jejunum. Radiat. Res. 38: 598-614.
Würgler, F.E. & B.E. Matter, 1969. Split-dose experiments with stage-14 oocytes of *Drosophila melanogaster*. Mutation Res. 6: 484-486.

Yamakawa, K. & A.H. Sparrow, 1965. Correlation of interphase chromosome volume and reduction of viable seed set by chronic irradiation of 21 cultivated plants during reproductive stages. Radiat. Bot. 5: 557-566.
Yamazaki, K., 1963. Zonal structure of shoot apices. In: Gamma Field Symposia (Ohmiya-machi, Ibaraki-ken) 2: 1-11.
Yuhas, J.M., 1968. The central nervous system syndrome of the mouse: effects of strain, age and conditioning exposure. Radiat. Res. 31: 714-721.