SYMMETRIC AND ASYMMETRIC HYBRIDIZATION IN *Citrus* spp.

A Dissertation

by

CLAUDINE BONA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2007

Major Subject: Horticulture

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ABSTRACT

Symmetric and Asymmetric Hybridization in *Citrus* spp.

(May 2007)

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The United States is the second largest producer of oranges and grapefruit. However, the US citrus industry experiences constraints in production due to pests, diseases and environmental concerns. Furthermore, due to the low diversity in current commercial scion cultivars any exotic diseases, if introduced into any of the producing states could be devastating. To maintain the US industry competitiveness it is necessary to improve cold, pest and disease resistance to allow expansion of citrus production areas in the US, and to improve fruit quality characteristics such as sweetness, vitamins and phytochemical contents and seedlessness. Sexual hybridization in most *Citrus* species is complicated because they are highly apomictic. Polyembryony makes it difficult to create large segregating populations for selection. Somatic hybridization by protoplast fusion circumvents sexual incompatibilities and is a powerful tool in genetic improvement. Symmetric and asymmetric hybdridization (gamma irradiation plus iodoacetamide) via protoplast fusion were performed with the objective of producing somatic hybrids of *Citrus paradisi* with *C. sinensis* and *C. reticulata* with *C. sinensis.*

These hybrids could be used for grapefruit improvement and to create genetic diversity. Furthermore, irradiated *Swinglea glutinosa* microprotoplasts were fused with 'Ruby Red' grapefruit and 'Mucott' tangor to assess the possibility of introgression of pieces of *S. glutinosa* chromosomes into the recipient protoplasts, a possible first step for radiation hybrid mapping. Double-inactivated fusions (irradiation + iodoacetamide) produced tetraploid and aneuploid plants, and hybridity was confirmed by amplified fragment length polymorphism (AFLP) analysis. This is the first report of obtaining rooted *Citrus* asymmetric hybrid plants, produced by irradiation plus iodoacetamide. AFLP confirmed presence of *S. glutinosa* into the receptor genomes, showing a possible donor introgression.

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INTRODUCTION

With a production of 105,431,984 metric tons (Mt) on 7,605,363 hectares in 2005, citrus is a very important crop worldwide. It is considered the most valuable fruit crop in international trade and has experienced continuous growth in the last decades, due mostly to consumer awareness of related health benefits (INFO COMM, 2006).

Brazil is the leading orange producer and the third largest producer of tangerines, mandarins and 'Satsuma' and The United States is the second largest producer of oranges and grapefruit. Together, these countries represent almost 70% of the total world production (FAOSTAT data, 2006).

The United States production is concentrated in California, Texas, Arizona and Florida, due to the tropical/subtropical characteristics of *Citrus* spp. which are usually frost-sensitive and require a humid environment and rich, well-drained soil for cultivation (INFO COMM, 2006; INFOAGRO, 2006). Besides environmental constraints, the US citrus industry experiences restraints on production due to many pests and diseases such as Citrus Tristeza Virus (CTV), Citrus blight, Greasy spot (*Mycosphaerella horii)*, Alternaria brown spot, Phytophthora-induced diseases, melanose (fungal), scab (*Elsinoe fawcetti* Bitanc.), citrus canker (*Xanthomonas axonopodis pv. Citri)*, postbloom fruit drop (PFD) (Colletotrichum acutatum) and in Florida, the major US producer, greening (*Diaphorina citri*) (Chung and Brlanski, 2006).

This dissertation follows the format and style of the Journal of the American Society for Horticultural Science.

Furthermore, due to the low diversity of scions currently in use, any exotic diseases, if introduced in any of the producer states, could be devastating.

In order to expand the US citrus production area it is necessary to improve salt, drought, and cold tolerance, as well as to improve pest and disease tolerance/resistance of the cultivars (Davies and Albrigo, 1994). Furthermore, to keep up with the public demand for fruit quality characteristics such as sweetness, good acidity balance, vitamin, phytochemical composition and seedlessness is essential to maintain the competitiveness of the industry. Higher yields, year-round availability and longer shelf-life would be additional benefits (INFOCOMM, 2006).

Grapefruit (*Citrus paradisi* Macf), sweet oranges (*C. sinensis* (L.) Osb.) and mandarins (*C. reticulata* Blanco) are the most commercially important citrus crops (Davies and Albrigo, 1994; Louzada et al., 2002). Grapefruit is a hybrid between pummelo and sweet orange. However, unlike pummelo, which produces zygotic embryos, grapefruit produces nucellar embryos, making breeding difficult. Also, its production and distribution is more limited than sweet oranges due to high heat requirements. Grapefruits, in general, present acid juice and moderately low total soluble solids (TSS) levels and are considered less palatable than oranges by many people. Sweet oranges are, in general, low to moderate in acids and moderate to high in per cent soluble solids. Hybridization between sweet oranges, including acidless oranges, and grapefruits could increase sweetness and decrease acidity of grapefruits. Likewise, hybridization between sweet oranges and mandarins could be of interest because it could

impart better color and increase cold-hardiness of sweet oranges and be benefic by imparting larger fruit sizes of sweet oranges (Davies and Albrigo, 1994).

Sexual hybridization in most *Citrus* species is, however, very complicated because of its complex reproductive biology. Most *Citrus* cultivars are very heterozygous and nucellar embryony is prevalent, particularly in sweet orange, grapefruit and lemon, and few important traits show single-gene inheritance patterns (Davies and Albrigo, 1994; Louzada et al., 2002; Ollitrault et al., 2000). Polyembryony impairs creation of large segregating populations for selection; therefore, achievement of desired characteristics is complicated even when using complementary parents. In addition, *Citrus* species have long juvenile periods. Hence, conventional breeding and selection are time-consuming (Grosser and Gmitter, 1990). Furthermore, sterility and sexual incompatibility are widespread in citrus.

Diversity in *Citrus* and related genera provides tremendous potential for developing hybrids with desirable characteristics. Such diversity has occurred during the long cultivation history of citrus due to weak barriers of reproductive isolation among species and nucellar embryony, which ensures intense vegetative reproduction in most *Citrus* species (Carvalho et al., 2005).

Despite this great variability within *Citrus* and related genera and their economical importance, most of the current cultivars originated from natural mutations in pre-existing cultivars, by chance selections, and by induced mutations, rather than from

breeding (Grosser and Gmitter, 1990). However, current demand does not allow researchers to rely solely on these methods.

Traditional breeding techniques are important and still used for citrus rootstock improvement and for mandarin improvement, however, unconventional techniques could help to shorten the breeding process.

Citrus has been genetically transformed (Dias, 1993), opening new opportunities for the development of novel citrus genotypes (Zhou et al., 2001). However, public antagonism, especially in Europe, towards such technologies has intensified interest in exploiting protoplasts in somatic hybridization and cybridization (Davey et al., 2005).

Somatic hybridization by protoplast fusion is a powerful tool in genetic improvement (Mendes et al., 2001). It brings together the genomes of two species (Schoenmakers et al., 1994) and can be used to transfer mono- or polygenic traits controlled by non-identified and non-cloned genes (Ramulu et al., 1996a,b). It circumvents sexual incompatibilities and offers the unique potential of simultaneously transferring nuclear and cytoplasmic genes. In somatic hybrids, dominant traits can be accumulated, irrespective of the heterozygosity level of the breeding material, and inbreeding depression is avoided. Seedlessness, another very important trait for citrus, can be induced by symmetric hybridization, by haploid + diploid fusion, and by cybridization to transfer cytoplasmic male sterility (CMS) (Calixto et al., 2004; Grosser and Gmitter, 2005; Liu et al., 1999; Ollitrault et al., 2000; Tian et al., 2002; Yamagishi and Glimelius, 2003; Zhou et al., 2001). Many distant citrus relatives which normally

could not be hybridized with *Citrus* due to sexual incompatibility, may serve as somatic parents and contribute as sources of abiotic and biotic resistance traits of interest (Ollitrault et al., 2000).

Many symmetric citrus somatic hybrids have been produced to data (Fu et al., 2003) and have been extensively used as tetraploid breeding parents (Guo et al., 2000) targeting seedlessness.

Asymmetric somatic hybridization (donor-recipient fusion or gamma fusion) using X- or γ-irradiation also has great potential because it allows partial genomic transfer from one cultivar to another (Derks and Colijn-Hooymans, 1989; Dudits et al., 1987). The contribution of the donor genome is minimized as chromosome elimination may be induced by the high radiation doses. Formation of donor irradiated colonies is avoided, as irradiation prevents donor parental escapes (Derks et al., 1992; Trick et al., 1994). Asymmetric hybrids containing only part of an irradiated genome would likely require fewer backcrosses to eliminate undesirable donor traits, and may expedite return to a near diploid level (Wijbrandi, 1989).

Iodoacetamide (IOA) is an irreversible inhibitor of enzymes involved in glycolysis (Epstein et al., 1981). Cells treated with it cannot divide and eventually degenerate (Bonnema and O'Connell, 1990). Added to protoplasts of the receptor genome, IOA facilitates the selection process of the created hybrids once only the truly hybrid cells are able to develop further due to genome complementation (Tian et al., 2002).

Protoplast culture and regeneration is well established in citrus, and symmetric somatic hybridization has been extensively performed (Grosser and Gmitter, 2005). The same cannot be said about asymmetric hybridization in citrus. Vardi et al. (1989) used donor-recipient protoplast fusion to produce cybrids. However, the first and only report on regeneration of citrus mixoploid hybrid plants via protoplast asymmetric fusion was published by Liu and Deng (2002) who produced asymmetric hybrids from Dancy tangerine and Page tangelo by using X-rays, yet plantlets were recalcitrant to root.

A different type of asymmetric hybridization was achieved by Louzada et al. (2002) who used microprotoplasts instead of irradiated protoplasts to produce asymmetric embryos with a few additional chromosomes. This work was based on the 'Microprotoplast Mediated Chromosome Transfer' (MMCT) technique, developed for mammalian cells by Fournier and Ruddle (1977) and efficiently adjusted for using in plants by Ramulu et al. (1996a,b), in which microprotoplasts holding one or few chromosomes, are produced.

Asymmetric somatic hybridization is also used for chromosome mapping (Wijbrandi, 1989; Yerle et al., 2004). A radiation dose breaks up chromosomes, and the resulting DNA fragments are rescued by hybridization with a background cell. Resulting hybrids, which typically retain only pieces of the target genome, are then assayed for those markers which are to be mapped (Tibshirani et al., 1999). Combination of gamma irradiation with MMCT could be interesting because chromosome breakage by radiation is prone to be more efficient in microprotoplasts than in protoplasts, not only due to the

smaller number of chromosomes but also because spindle formation has been inhibited by chemicals and microtubules-toxins used in the microprotoplasts preparation (Zhang et al., 2006). Hence, there is a better chance of more breakage and therefore, a more efficient introgression of the donor chromosome pieces in the receptor genome. The applicability and effectiveness of radiation hybrid mapping in plants has been demonstrated (Gao et al., 2004,2006). Same principles could be extended to *Citrus.*

The primary goal of this research was to generate diversity by creating somatic symmetric and asymmetric hybrids using sweet oranges, mandarins and grapefruits, the most important commercial citrus via protoplast fusion with gamma irradiation for asymmetry and in conjunction with IOA for a double genome inactivation and more efficient selection.

A secondary goal was to observe the potential of combining MMCT and gamma irradiation for introgression of donor chromosome pieces into a background cell as a possible first step for future use in radiation mapping.

LITERATURE REVIEW

World production of citrus fruit has experienced continuous growth because consumption of citrus has increased not only as fresh fruit but also as juice. This has resulted from preferences for convenient and healthy products, improvements in quality, competitive prices, promotional activity and technological advances in processing, storage and packaging. This increase has boosted citrus juice production and international juice trade. The main citrus fruit producing countries are Brazil, the Mediterranean countries, the United States (where fruits for the fresh market are mainly grown in California, Arizona and Texas, while for processing are mostly produced in Florida) and China (INFO COMM, 2006).

The US citrus industry, however, faces some economic constraints on its production. Citrus production areas are threatened by pests, diseases, and environmental problems, to which current commercial rootstock and scion cultivars are susceptible. Furthermore, the US citrus industry is experiencing a decrease in market share due to competition from foreign producers in both fresh and processed markets (Bowman et al., 2004). Dealing with these deficiencies requires creation of diversity through development of new varieties with characteristics such as freeze-hardiness, disease tolerance/resistance, as well as, because of consumer preferences, seedless fruits, well nutritious value and sweetness. This requires the development of new scion varieties which are competitive on the world market and are available over a long production season (Bowman et al., 2004; Davies and Albrigo, 1994).

Unfortunately, *Citrus* species present a complex reproductive biology. Citrus seedlings have long juvenile periods, ranging on average from five to 15 years, another complicating factor for conventional breeding. Such a characteristic makes breeding costly (Davies and Albrigo, 1994; Grosser and Gmitter, 1999). Many *Citrus* species and genotypes display various degrees of pollen or ovary sterility. Most *citrus* cultivars are highly heterozygous, and few important traits show single-gene inheritance patterns (Louzada et al., 2002). When crossing fertile individuals, the resulting offspring is typically variable and replete with unexpected and undesirable types. Heterozygosis may also promote inbreeding depression because deleterious recessives have the opportunity to combine by meiotic recombination (Deng et al., 2000, Furr et al., 1969; Grosser and Gmitter, 1999).

Nucellar embryony is prevalent, particularly in sweet orange, grapefruit and lemon (Davies and Albrigo, 1994; Ollitrault et al., 2000), and sexual embryos often die for lack of nourishment, making the construction of large segregating population difficult (Grosser and Gmitter, 1999; Louzada et al., 2001). Polyembryony greatly impairs the creation of large segregating populations for selection of desired characteristics even when using complementary parents.

Some alternative possibilities to increase genetic diversity other than conventional breeding exist. Diversity has been obtained by selection of naturally occurring mutations, as well as by induced mutations (Grosser and Gmitter, 1990). Mutation breeding has been extensively used to induce mutation in citrus, and some important selections have been produced, such as ‘Star Ruby’, an induced seed mutation from

‘Hudson’ grapefruit and ‘Rio Red’, the main variety grown in Texas and Mexico, produced from the non-commercial A&I 1-48, which was produced by irradiation of a nucellar “Ruby Red’ budwood (Graça and Louzada, 2006, unpublished). However, the main difficulty in mutation breeding of vegetatively propagated plants is that mutations are one-cell events and plant parts, with their multicellular tissues, are thus automatically chimeric. Recovery of non chimeric products that can be effectively screened is difficult (Broertjes and Keen, 1980; Lee, 1988).

Recombinant DNA technologies have been explored in citrus (Almeida et al., 2003; Frydman et al., 2004; Guo et al., 2005; Peña et al., 2004), however, public antagonism, especially in Europe, towards such technologies has re-intensified interest in exploiting protoplasts in somatic hybridization, cybridization, proteomics and metabolomics (Davey et al., 2005). Transformation efficiency in citrus is, in any case, generally low. Some species are non transformable and in transformable species, more than 60% of produced shoots are escapes and high frequency of chimeras is common (Domingues et al., 1999). Furthermore, availability of horticulturally important genes is scarce (Louzada et al., 2002).

Another possibility for genetic manipulation is somatic hybridization. Somatic hybridization or protoplast fusion is a powerful tool in genetic improvement and creation of diversity in gene pools, because, it not only overcomes sexual barriers between species but also allows combination of nuclear, chloroplastic and mitochondrial genomes in new patterns (Mendes et al., 2001; Ollitrault et al., 2000).

SOMATIC HYBRIDIZATION

Somatic hybridization by protoplast fusion can bring together the genomes of two species (Schoenmakers et al., 1994), and transfer mono- or polygenic traits controlled by unidentified and uncloned genes (Ramulu et al., 1996a,b). It circumvents sexual incompatibilities and offers the unique potential of simultaneously transferring nuclear and cytoplasmic genes (Grosser and Gmitter, 1999).

In somatic hybrids, dominant traits can be accumulated, irrespective of the heterozygosity level of the breeding material. Inbreeding depression is avoided because there is no haploidization of the recipient genomes and deleterious recessives alleles do not have the opportunity to combine (Calixto et al., 2004; Liu et al., 1999; Ollitrault et al., 2000; Tian et al., 2002; Yamagishi and Glimelius, 2003; Zhou et al., 2001). The unmasking of deleterious recessives by meiotic segregation, potentially expressed as inbreeding depression, does not occur with somatic hybridization.

Somatic hybridization via protoplast fusion may allow creation of novel genotypes by combining different species or cultivars. Some cultivar combinations previously considered impossible to perform can be done using protoplast fusion. Related genera of the *Citrae* and *Clauseneae* tribes, which normally cannot be hybridized with citrus due to sexual incompatibility, can serve as sources of abiotic and biotic resistance traits of interest (Ollitrault et al., 2000).

Combinations of parents with complementary favorable traits and/or presenting superior genotypes can be performed, irrespective of sexual incompatibility, sterility or polyembryony, without disrupting favorable gene combinations. Furthermore, traits that

are conditioned by dominant alleles in one of the donors should be expressed in the somatic hybrid (Grosser and Gmitter, 1999).

SYMMETRIC SOMATIC HYBRIDIZATION IN CITRUS

Since the first citrus somatic hybrid was obtained by Ohgawara et al. (1985) via protoplast fusion of *C. sinensis* Osb. and *Poncirus trifoliata*, somatic hybridization has contributed tremendously to citrus improvements. Several citrus somatic hybrids have been reported to be in use in various breeding programs. Many achievements were made by somatic hybridization (Fu et al., 2003). Louzada et al. (1993) first reported production of hybrid plants between two sexually incompatible *Citrus* genera via protoplast fusion by fusing *C. sinensis* (L.) Osbeck cv. 'Hamlin' with *Atalantia ceylanica* (Arn.) Oliv.. Grosser et al. (1996) combined *Citrus* with seven related genera, some of them sexually incompatible, via protoplast fusion for rootstock improvement. Guo et al. (2000) fused protoplasts of 'Bonnanza' navel orange (*C. sinensis*) with Red Blush grapefruit (*C. paradisi*). The regenerated plants flowered precociously. Mendes-da-Gloria et al. (2000) obtained plants from protoplast fusions of 'Rangpur' lime (*C. limonia* L. Osb.) and 'Caipira' sweet orange (*C. sinensis*), aiming to combine the drought tolerance and vigor from the 'Rangpur' lime with the blight tolerance of 'Caipira' sweet orange. Deng et al. (2000) produced more than 20 interspecific, intergeneric, and intertribal somatic hybrids, as well as putative hybrids. Fu et al. (2003) regenerated plants from protoplast fusions of 'Newhall' navel orange (*C. sinensis*) and 'Chicken Heart' sweet wampee (*Clausena lansium*). Calixto et al. (2004) produced hybrids of 'Hamlin' sweet orange and

'Singapura' pummelo with potential to be used as blight, Citrus tristeza virus (CTV), and *Phytophthora*-induced disease tolerant rootstocks. Khan and Grosser (2004) fused *C. micrantha*, a progenitor of lime, with sweet orange (*C. sinensis*) to recreate a lime-like fruit using the sweet orange as source of resistance against Witches' broom disease of lime (WBDL), reporting for the first time the use of a progenitor species in somatic hybridization experiments. Takami et al. (2004) produced intergeneric somatic hybrids between 'Kumquat' (*Fortunella japonica* Swingle) and 'Morita' navel orange to introduce seedless kunquats for the Japanese market. Wu et al. (2005) used protoplast fusion to produce novel allotetraploid mandarin hybrids for use as parents in crosses with diploids to produce easy-peel, seedless, triploid *Citrus* cultivars and Takami et al. (2005) utilized intergeneric somatic hybrids as index discriminating taxa for *Citrus* and related species.

In addition to the examples cited above, several inter- and intra- specific and inter-generic and inter-tribal symmetric somatic hybrids have been produced by Liu and Deng (2000), Saito et al. (1991), Takami et al. (2004) and Wu et al. (2005), as well as many cybrids (cytoplasmic hybrids) by Cabasson et al. (2001), Guo et al. (2004, 2006), Liu et al. (2002), Saito et al. (1993), Xu et al. (2004). A more complete list of produced symmetric somatic hybrids and cybrids is found in Calixto (2003).

Seedless fresh fruit varieties have been developed by symmetric hybridization, haploid + diploid fusion and targeted cybridization to transfer cytoplasmic male sterility (CMS). The male sterility is an important agronomic trait controlled at least partially by the mitochondrial genome. Cybridization is also interesting in that it offers the

possibility of manipulating chloroplast and mitochondrial genomes and evaluating their role on cultivar qualities in citrus (Cabasson et al., 2001) were, if not for the ability of protoplast fusion to yield such cytoplasmic-nuclear products, many years of repetitive sexual backcrossings would be required to produce the cytoplasmic substitutions (Grosser and Gmitter, 2005; Guo et al., 2004).

Symmetric somatic hybridization has allowed development of inter- and intra-generic allotetraploid hybrids, even when sexually incompatible parents were used for protoplast isolation. Such genotypes are very important for rootstock improvement, as they hybridize complementary genotypes without breaking up successful gene combinations and yet provide an opportunity to introduce disease and pest resistance, better adaptation to specific soil and climate niches, and for tree size control (Grosser and Gmitter, 2005; Louzada et al., 1993; Mendes-da-Gloria et al., 2000; Wu et al., 2005).

Wide somatic hybridizations make it possible to combine *Citrus* species with sexually incompatible genera that possess desirable attributes (Grosser and Gmitter, 2005). Wild relatives are potential sources of useful resistance traits for citrus improvement (Fu et al., 2003) and represent a largely untapped reservoir of genetic diversity. Somatic hybridization of wild relatives with *Citrus* species may allow better horticultural performance for the wild relatives and make possible the use of resulting somatic hybrids as rootstocks (Louzada and Grosser, 1994). For example, somatic hybrids have been produced among *Citrus* and *Clausena* (subtribe Clausineae),

Citropsis, *Severinia* and *Atalantia* (subtribe Citrinae) and *Feronia* (subtribe Balsamocitrinae) (Louzada and Grosser, 1994).

Even though symmetric somatic hybrids have great potential for rootstock improvement, they may not have direct application as scion cultivars (Louzada et al., 2002). Most of the available hybrids are allotetraploids and may not be directly used as commercial scion cultivars because they contain genomes of both fusion parents. As a consequence, they express both the desirable and undesirable traits simultaneously, which, to some degree, limits the utilization of such somatic hybrids (Liu and Deng, 2002). Hybrids created by symmetric hybridization may present complex genetic constitution and require many backcrosses to establish new cultivars, and may also present chromosome instability and sterility. Furthermore, no significant benefits have been presented for the two most important citrus commercial fruits, grapefruits and sweet oranges (Louzada et al., 2001).

ASYMMETRIC HYBRIDIZATION

Asymmetric hybrids, those with fewer genes from one partner than from the other, may arise from spontaneous chromosome elimination in some distant combinations. However, in such hybrids, there is no way to manipulate the amount of elimination or to direct which genome will undergo chromosome elimination (Hinnisdaels et al., 1991). Another way to achieve alien chromosome introgression is by breakage and fusion of chromosome fragments by radiation, i.e. X- or γ-rays (Chang and Jong, 2005). Elimination of chromosomes may also be induced as result of inactivation of mitotic

capacity in one partner by chemicals, such as colchicine (Harms, 1992; Sanamyan and Rakhmatullina, 2003). Wu and Mooney (2002) produced three desirable non-chimeric, autotetraploid plants of the mono-embryonic tangor cultivar ‘Umatilla’ using 0.05% colchicine and one from 0.1% colchicine and one mixoploid ‘Dweet’ plant by using 0.1% colchicine.

Irradiation may have the potential to direct the process of chromosome elimination (Hinnisdaels et al., 1991), since elimination seems to be dose-dependent (Wijbrandi, et al., 1990). Hence, asymmetric hybrids are prone to have fewer genes from one partner than from the other since the contribution of the donor genome is minimized as chromosome elimination is induced by radiation doses (Liu and Deng, 2002; Trick et al., 1994). Liu and Deng (2002) observed that regeneration of shoots from citrus interspecific hybrids was dose dependent. Trick et al. (1994) using tobacco hybridization as a model, found that radiation-induced elimination of donor chromosomes increased with gamma dose and that in long-term callus culture donor-chromosome elimination was a variable process. Nevertheless, correlation between irradiation dose and number of donor chromosomes in the hybrid cells has been controversial (Derks et al., 1992).

The dose of irradiation, the ionizing-radiation amount absorbed per unit mass, is presented as gray (Gy), which is the standard unit of absorbed ionizing-radiation dose and is equivalent to one joule per kilogram. One gray corresponds to 100 rads (common unit of radiation) (Ahloowalia and Maluszynski, 2001; Kondoh et al., 1998).

Ionizing radiation causes fragmentation of DNA and induces both single and double-strand breaks, which lead to elimination of donor chromosomes, formation of

micro-chromosomes and translocations (Derks et al., 1992). The chromosome breakage in the irradiated cells seems not to be random (Fernandez et al, 1990). Wijbrandi (1989) observed that three donor *Lycopersicum peruvianum* loci, located on the chromosomes 2, 4 and 7, were present in each of the resulting asymmetric hybrids, suggesting linkage.

Asymmetric somatic hybridization (donor-recipient fusion or gamma fusion) using X- or γ-irradiation allows partial genomic transfer from one cultivar to another (Derks and Colijn-Hooymans, 1989; Dudits et al., 1987). Partial genome transfer is achieved by irradiation of the donor protoplasts to induce fragmentation and subsequent elimination of chromosomes before fusion with non-irradiated receptor protoplasts (Derks et al., 1992; Liu and Deng, 2002; Trick et al., 1994). Contributions by the donor genome are minimized, i.e., chromosome elimination increases with higher radiation doses. Asymmetric hybrids containing reduced representation from the donor genome would theoretically require fewer backcrosses to eliminate undesirable traits and re-establish a near diploid level (Wijbrandi, 1989). Besides that, partial genome transference can be better tolerated than the whole donor genome (Ramulu et al., 1996a,b).

Asymmetric hybrids have been produced in fusing species of Medicago, tobacco, tomato, potato, tomato + potato, *Arabidopsis thaliana* + *Brassica napus* and rice + *Zizania latifolia* (Liu et al., 1999; Tian et al., 2002).

Asymmetric hybridization would seem to offer a great potential for genetic improvement of citrus. However further research is needed. Vardi et al. (1989) produced cybrids by donor-recipient protoplast-fusion. However, the first and only report about regeneration of mixoploid hybrid plants via protoplast asymmetric fusion in citrus was

published by Liu and Deng (2002) who produced asymmetric hybrids from Dancy tangerine and Page tangelo by using X-rays. However, plants were recalcitrant to rooting in rooting inducing media and had to be grafted.

Another type of somatic hybridization capable of generating diversity is 'Microprotoplast Mediated Chromosome Transfer' (MMCT), developed for mammalian cells by Fournier and Ruddle (1977). Micronucleation may be induced by prolonged mitotic arrest by using microtubule inhibitor compounds (Fournier and Ruddle, 1977). Fournier (1981) produced micronucleated mouse L-cells with colcemid. Falconier and Segull (1987) noticed that that the herbicide amiprophos-methyl (APM) was more efficient than colchicine for plant microtubule depolymerization. The micronuclei formed after the prolonged mitotic arrest can be physically isolated. When protoplasts are exposed to cytochalasin B under high speed centrifugation, the nucleus, some surrounding cytoplasm and the plasma membrane are pinched off to form subprotoplasts, some containing nuclei and others lacking them (Thomas et al., 1976; Wallin et al., 1977). The fusion of potato microprotoplasts with tobacco and tomato protoplasts by MMCT using APM are reported by Ramulu et al. (1996a,b)

In citrus, the MMCT technique was first applied by Louzada et al. (2002) who fused hydroxyurea (HU) treated microprotoplasts of 'Ruby Red' grapefruit containing one to three chromosomes with protoplasts of 'Succari' sweet orange and *S. glutinosa* microprotoplasts with protoplasts of sour orange. They obtained embryos and suspension cells with a few additional chromosomes.

It has been shown that transfer of small portions of the genome (1-2) chromosomes together with a small portion of cytoplasm (a thin layer near the micronuclei) significantly reduce the destabilization of the acceptor cell (Yemets and Blume, 2003) and such generated cell lines are especially powerful gene manipulation tools (Fournier, 1981; Fournier and Ruddle, 1977).

Asymmetric somatic hybridization may also be used for chromosome mapping (Wijbrandi, 1989). Irradiation resultant small fragments of chromosomes greatly increase the power of physical mapping in the species of interest (FAO, 2006). Radiation hybrid mapping is a powerful tool for mapping genomes and it's applicable to any species for which somatic hybrid cells can be made and provides a most efficient route to the production of ordered maps containing nonpolymorphic or minimally polymorphic markers (Womack, 1999). The technique is based in that if two markers are further apart on the chromosome, the more likely a given irradiation dose will break the chromosome between them, placing the markers on two separated chromosomes. By estimating the frequency of breakage, and thus the distance between markers, it is possible to determine their order in a manner analogous to meiotic mapping (Cox et a., 1990).

Radiation hybrid mapping was originally developed for animal systems but may also be used in plants (Wardrop, et al., 2002). Riera-Iizarazu et al. (2000) irradiated an oat-maize monosomic addition line containing chromosome 9 and produced maize chromosome 9 radiation hybrids, and oat lines possessing different fragments of maize chromosome 9. Kynast et al. (2002) developed a complete set of oat-maize chromosome

additions in order to map maize sequences and to study expression of maize genes in the genetic background of oats. Wardrop et al. (2002) generated and cultured *in vitro* plant (barley) radiation hybrids but could not create a map from them. Gao et al. (2004,2006), however, were able to create asymmetric sexual interspecific hybrids and successfully create RH maps. The results indicated genome-wide RH mapping is quite feasible in cotton.

The combination of gamma-irradiation with MMCT may allow the insertion of donor chromosome pieces inside the receptor genome, which would be a first step in creating radiation hybrid cells because, in spite of its small size (haploid genome size has approximately 385 Mb (Gmitter et al., 1999), currently there are no physical maps of the *Citrus* genome (Roose et al., 2000; USDA, 2006).

S. glutinosa, in the Balsamocitrinae subtribe, pursues a very distinct, heterochromatin-poor karyotype and presents the smallest chromosomes known in the whole sub-family *Aurantioideae* (Guerra et al., 2000). For such distinct characteristics and for being a distant citrus relative, ‘Swinglea’ irradiated microprotoplasts would be a great candidate for fusion with ‘Ruby Red’ grapefruit because it is a very distant relative of *Citrus*, belonging to a different sub-tribe, which could facilitate identification of possible ‘Swinglea’ DNA insertions by band polymorphism using AFLP.

CONFIRMATION OF HYBRIDITY

Another advantage of asymmetric hybridization over symmetric hybridization is that, by irradiation of the donor protoplasts, selection of hybrids is made easier because

formation of irradiated colonies is avoided, as irradiation prevents donor parental escapes (Derks et al., 1992; Trick et al., 1994). Furthermore, receptor protoplasts may be treated with iodoacetamide (IOA) (Liu et al., 1999; Tian et al., 2002), which is a metabolic inhibitor (Epstein *et al*, 1981). Cells treated with IOA cannot divide and eventually degenerate (Bonnema and O'Connel, 1990; Liu et al., 1999; Varotto et al., 2001). Such double inactivation, using IOA inactivated receptors and gamma-irradiation inactivated donors, makes it possible to enhance selection performance by avoiding possible donor and receptor parental escapes and facilitating hybrid identification, since only the truly hybrid cells or superior mutants are able to develop further due to genome compensation (Tian et al., 2002).

Confirmation of hybrid status has been done in the great majority of studies by morphology observation, chromosome number, flow cytometry and by random amplified polymorphic DNA (RAPD) analysis (Costa et al., 2004; Deng et al., 2000; Grosser et al., 1996; Khan and Grosser, 2004; Louzada and Grosser, 1994; Mendes et al., 2001; Mendes-da-Gloria et al., 2000; Takami et al, 2005; Wu et al., 2005). Isoenzyme analysis was extensively used for hybrid characterization. However, hybrid tissues may possess isoenzymes band profiles characteristic of each parent, as well as additional bands which may be either a signal of hybridity or artifacts (Lynch et al., 1993). Chromosome counting from actively dividing cells, as from root tips and growing apices, help in the confirmation of hybridity. However, they may be inaccurate due to possible doublings, elimination of chromosomes, breakage or overlapping of chromosomes during slides preparations that may confuses accurate chromosome

number counting (Lynch et al., 1993). Accordingly with Kitajima et al. (2001), citrus chromosome preparations may be prepared using root tips or leaves, but aerial tissues are more desirable for accurate cytological and karyotyping studies in heterozygous fruit trees. Andras et al. (1999) suggested that a drop-spreading technique is recommended to produce cytoplasm-free preparations from plants with small chromosomes. Ploidy analysis by flow cytometry has been very useful to complement characterization of hybrids (Fu et al., 2004).

Nevertheless, molecular markers for detection of alien DNA, e.g., restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) amplified fragment length polymorphism (AFLP) and variable numbers of tandem repeats (VNTR) are more reliable (Karp et al., 1996). RAPD markers are used most commonly, one of the reasons is that the method is simple and inexpensive (Oliveira et al., 2004). However, RAPD was failed to differentiate between sweet orange varieties, so Targon et al. (2000) suggested more sensitive methods of analysis.

Cybrids have been detected mostly by cleaved amplified polymorphic sequence (CAPS), chloroplast simple sequence repeat (cp-SSR) and mitochondrial restriction fragment length polymorphism (mt-RFLP) (Cabasson et al., 2001; Guo et al., 2004,2006; Takami et al., 2004; Vardi et al., 1989; Xu et al., 2005). Both RFLP and AFLP have been useful in detecting chromosome losses and in revealing more information about hybridity in somatic hybrids, because provide reliable markers, high resolution and efficiency (Fu et al., 2004).

AFLP is a DNA fingerprinting technique which is a very powerful tool for distinguishing closely related cultivars (Hanada et al., 2003). Using this method, sets of restriction fragments may be visualized by PCR without knowledge of nucleotide sequence (Vos et al., 1995). AFLP markers are highly polymorphic and reproducible (James et al., 2003) and the technique is robust and reliable (Vos et al., 1995).

Pang et al. (2006) revealed genetic diversity of *Poncirus* accessions by AFLP which has been reported to be highly polymorphic in citrus (Campos et al., 2005; Chao et al., 2005).

AFLP has proven to be robust against methylation alterations. Shaked et al. (2001) used AFLP and methylation-sensitive amplification polymorphism (MASP) to obtain a quantitative estimate of the timing and frequency of allopoliploid-associated genetic and epigenetic response of wheat in wide hybridizations and obtained reproducible patterns of elimination which were proven not to be attributed to heterozygosity or methylation. They concluded that AFLP is a robust and high-throughput means to assess the induction of genomic rearrangements. Furthermore, genetic and epigenetic evaluations of citrus calluses performed by Hao et al. (2004) using MSAP suggested that ploidy level remains stable during long *in vitro* periods.

Genomic *in situ* hybridization (GISH) is a technique which provides a direct and visual method for effective number and position determination of the parental chromosomes (Fu et al., 2004), makes possible visualization of alien chromosomes and was successfully applied to identify citrus somatic hybrids by Fu et al. (2004) and Guo et al. (2004). There are two reasons why only a few GISH studies have been performed in

citrus somatic hybrids: citrus chromosomes are small, 2μ in size (Usman, 2005), and morphologically undistinguishable, and the GISH method has several key steps affecting the final result. Researchers must be highly skilled to successfully obtain high-quality chromosome slides, since to have well-dispersed chromosomes is an important factor (Fu et al., 2004).

Relevance of this work. The importance of this work is that **s**omatic hybridization is a powerful tool to generate diversity. It offers possibilities such as **c**reation of potentially high-quality citrus hybrids, production of tetraploids to be used in interploid crosses and physical mapping of the genomes.

Gamma-irradiation of donor protoplasts before fusion causes chromosome breakage, and allows part of a donor genome to be inserted into the recipient. Nucleus transference of less-than-entire genomes may reduce the number of backcrosses needed to eliminate possible undesirable alien traits.

Furthermore, hybridization of grapefruits with sweet oranges could be very important to scion improvement and diversity, where the creation of such hybrids via conventional breeding would be practically impossible.

Use of *S. glutinosa* microprotoplast and irradiation before fusion with grapefruit or ‘Murcott’ protoplasts may facilitate introgression of pieces from the donor into the receptor, which, depending on future stability studies, could be a first step towards citrus radiation mapping and comprehensive genome sequencing.

OBJECTIVES

- Create somatic symmetric and asymmetric hybrids using sweet oranges, mandarins and grapefruits with potential for scion improvement and tetraploid production for use in interploid crosses resulting in seedlessness.
- Determine the potential of combining MMCT and gamma irradiation for asymmetric somatic hybridization in *Citrus*.
- Evaluate the possibility of using asymmetric somatic hybrids in Citrus breeding and genomic (mapping).

MATERIALS AND METHODS

PROTOPLAST SOURCE

Protoplasts were isolated from habituated embryogenic suspension cells of the grapefruit cultivars ‘Ruby Red’ and ‘Flame’ (*C. paradisi* Macf.), the sweet oranges ‘Itaborai’, ‘Natal’, Valencia’ and ‘Succari’ (*C. sinensis* (L.) Osbeck), from ‘Satsuma’ mandarin (*C. reticulata* Blanco) and from ‘Murcott’ tangor (Murcott Honey, Smith) (*C. reticulate* × *C. sinensis*).

Suspension cells, produced from ovule-derived embryogenic callus, kindly provided by J. W. Grosser (Citrus Research and education Center, University of Florida, Lake Alfred), were maintained in a two-week subculture cycle in liquid half-strength H+H medium (Appendix A) under constant agitation on a horizontal gyratory shaker (Lab-Line, USA) at 130 rpm, at room temperature and under constant illumination (two growth lux lamps of 20 W each (GE lighting, Nela Park, Cleveland, OH) (Grosser and Gmitter, 1990; Louzada et al., 2002).

Protoplast isolation. The protocol for protoplast isolation was adapted from Grosser and Gmitter (1990). Approximately 1 gram of fresh weight drained cells (4 to 10 days after subculturing) was placed in a 5 cm diameter Petri dish and 0.5 to 1 mL of enzyme solution plus 4 to 5 mL of 0.4 to 0.7 M BH3 medium (Appendix B) were added (Grosser and Gmitter, 1990). Cells were digested overnight, in the dark, on a rocker platform (Bellco Glass, Inc, Vineland, NJ) with 6 oscillations per minute.

To improve protoplast isolation, different ratios of 0.6 M BH3-enzyme solution were attempted. Furthermore, for some cultivars, a two-step digestion was performed.

The enzyme solution consisted of 1% cellulase R-10 (Karlan, Santa Rosa, CA), 0.2% pectolyase Y-23 (Karlan, Santa Rosa, CA), 1% macerozyme R-10 (Karlan, Santa Rosa, CA) 0.024 M $CaCl_2$, 0.92 mM NaH_2PO_4, 6.15 mM 2-[N-morpholino]ethane sulforic acid (MES) (Sigma, Dallas, TX), and 0.4 to 0.7 M of mannitol. The pH was adjusted to 5.6, and the solution was filter-sterilized.

Protoplasts were separated from the debris by filtering through a sterile 45 μm mesh stainless steel sieve, transferred to sterile 15-mL centrifuge tubes, and centrifuged at 100 g_n for 5 min. The supernatant was removed and the protoplast pellet was carefully ressuspended in 5 mL of 25% sucrose. Mannitol (13%) was slowly added to form a gradient, and the tubes were centrifuged for 5-10 min at 100 g_n. The protoplast band was carefully removed with a Pasteur pipette and transferred to clean tubes. Protoplasts were washed with 5 mL of liquid BH3 medium and centrifuged for 5 min at 100 g_n. Protoplast pellets were diluted in a small volume of BH3 to approximately 1×10^6 protoplast.ml^{-1} (Grosser and Gmitter, 1990). For cell counting, a Bright-Line® hematocytometer (Hausser Scientific, Horsham, PA) was used.

SOMATIC HYBRIDIZATION

Protoplast fusion was performed based on the polyethylene glycol (PEG) method described by Grosser and Gmitter (1990). Fusions were performed using different cultivar combinations (Table 1). Approximately equal amounts of the two kinds of

protoplasts were mixed. Two or three drops of mixed protoplasts were placed in the center of 5-cm diameter plates. Two drops of PEG solution (Appendix C) were added, one at a time, and protoplasts were allowed to fuse for 10 min. Two drops of 9:1 A:B solution (Appendix D,E) were added, one at each side of the fusion drop, and incubated for 15 min. The protoplasts were washed from the fusion solution by 12 drops of BH3 medium, placed around the protoplasts, and left for 5 min, followed by three additional washings of 10 min each. Normal protoplast-protoplast fusions, i.e, without any prior treatment, will be designated as (P-P).

ASYMMETRIC HYBRIDIZATION

Donor-protoplast irradiation. Fusions were performed using randomly chosen donor-receptor combinations (Table 1). Non-fused irradiated protoplasts were plated for control. Donor protoplasts were irradiated at the USDA/APHIS Moore Air Base, Edinburg, TX. Protoplasts were exposed to gamma ray doses of 30, 50, 70, 80, 100, 150, 200 or 300 grays [1 kilorad (Krad) = to 10 grays (Gy)], prior to fusion with receptor protoplasts. Normal protoplast-irradiated protoplast fusions will be designated as (P-I).

Iodoacetamide treatment. Double inactivation was achieved by treating receptor protoplasts with 3 mM iodoacetamide (IOA) (Sigma, Dallas, TX) for 10, 13, 15 or 20 min (Bonnema and O'Connell, 1990. Protoplasts were washed with liquid BH3 and centrifuged for 5 min at 100 g_n. The pellet was re-suspended in fresh liquid BH3 medium, and the treated protoplasts were fused with the irradiated donor protoplasts.

Fusions were performed using randomly chosen donor-receptor combinations (Table 1). Non-fused irradiated protoplasts and IOA treated protoplasts. Non-fused protoplasts were plated as controls. IOA treated protoplast-irradiated protoplast fusions will be designated as (IOA-I).

Table 1. Somatic symmetric and asymmetric fusions of citrus protoplasts.

Protoplast + protoplast fusion (P-P)	**Protoplast + irradiated protoplast fusion (Grays) (P-I)**	**3mM IOA treated protoplast (min) + irradiated protoplast fusion (Grays) (IOA-I)**
‘Ruby Red’ + ‘Succari’	‘Ruby Red’ + ‘Natal’ (100)	‘Ruby Red’ (20) + ‘Satsuma’ (150)
‘Ruby Red’ + ‘Itaborai’	‘Ruby Red’ + ‘Succari’ (100)	‘Ruby Red’ (20) + ‘Succari (150)
‘Ruby Red’ + ‘Natal’	‘Ruby Red’ + ‘Itaborai’ (100)	‘Ruby Red’ (20) + ‘Natal’ (100)
‘Ruby Red’ + ‘Valencia’	‘Ruby Red’+ ‘Natal’ (150)	‘Ruby Red’ (20) + ‘Natal’ (200)
‘Flame’ + ‘Succari’	‘Ruby Red’ + ‘Itaborai’ (150)	‘Ruby Red’ (20) + ‘Natal’ (300)
‘Flame’ + ‘Natal’	‘Ruby Red’ + ‘Satsuma’ (150)	‘Ruby Red’ (20) + ‘Satsuma’ (200)
‘Flame’ + ‘Itaboraí’	‘Ruby Red’ + ‘Natal’ (80)	‘Ruby Red’ (20) + ‘Succari’ (150)
	‘Ruby Red’ + ‘Murcott’ (70)	‘Ruby Red’ (20) + ‘Succari’ (50)
	‘Ruby Red’ + ‘Natal’ (70)	‘Ruby Red’ (20) + ‘Natal’ (50)
	‘Flame’ + ‘Itaboraí’ (100)	‘Ruby Red’ (20) + ‘Succari’ (100)
	‘Flame’ + ‘Natal’ (100)	‘Ruby Red’ (20) + ‘Itaborai’ (100)
	‘Flame’ + ‘Succari’ (100)	‘Ruby Red’ (20) + ‘Itaborai’ (80)
	‘Flame’ + ‘Satsuma’ (100)	‘Ruby Red’ (20) + ‘Succari’ (80)
	‘Flame’+ ‘Satsuma’ (150)	‘Ruby Red’ (15) + ‘Murcott’ (100)
	‘Natal’ + ‘Ruby Red’ (150)	‘Ruby Red’ (15) + ‘Changsha’ (100)
		‘Ruby Red’ (15) + ‘Itaborai’ (100)
		‘Ruby Red’ (15) + ‘Succari’ (100)
		‘Ruby Red’ (15) + ‘Natal’ (100)
		‘Ruby Red’ (15) + ‘Natal’ (50)
		‘Ruby Red’ (15) + ‘Itaborai’ (50)
		‘Ruby Red’ (15) + ‘Succari’ (50)
		‘Ruby Red’ (15) + ‘Murcott’ (30)
		‘Ruby Red’ (15) + ‘Succari’ (30)
		‘Ruby Red’ (10) + ‘Natal’ (50)
		‘Ruby Red’ (10) + ‘Murcott’ (50)
		‘Ruby Red’ (10) + ‘Changsha’ (50)
		‘Flame’ (20) + ‘Natal’ (150)
		‘Flame’ (20) + ‘Succari’ (100)
		‘Flame’ (20) + ‘Itaborai’ (150)
		‘Murcott’ (20) + ‘Natal’ (100)
		‘Murcott’(20) + ‘Itaborai’ (100)
		‘Murcott’ (15) + ‘Natal’ (50)
		‘Murcott’ (15) + ‘Succari’ (50)
		‘Murcott’ (10) + ‘Itaborai’ (100)
		‘Natal’ (15) + ‘Ruby Red’ (50)
		‘Itaborai’ (15) + ‘Murcott’ (50)

PROTOPLAST CULTURE

The fused protoplasts were cultured in six drops of BH3 and 12 drops of the same medium were added to avoid protoplast desiccation.

Plates were sealed and stored in the dark, at room temperature until microcalli started to form. Osmotic stress was gradually reduced by adding three to four drops of 1:1:1 and later 1:2 liquid media which are mixtures of BH3 and EMEP media (Appendices F and G). Microcalli colonies were transferred to solid EMEP (Appendix H) medium and gradually exposed to light (Grosser and Gmitter, 1990).

The whole process (protoplasts isolation, before and after irradiation, fusion, cell wall formation, and cell division) was monitored under a Nikon Eclipse TE300 inverted microscope (Nikon Instruments Inc. Melville, NY) and images were captured by using the Image-Pro® Plus software version 4.5.1 in a CoolSNAP-PROcf camera (Media Cybernetics, Silver Spring, MD).

Formed embryos were transferred and sub-cultured on fresh solid EMEP until they reached approximately 0.5 cm, then transferred to 1500 media (Appendix I) for further development.

Plantlet regeneration and root induction. Well developed embryos were transferred to B+ embryo germination medium (Appendix J) for shoot formation, while poorly developed ones were transferred to DBA3 media (Appendix K) media for shoot induction.

Proliferating embryos and embryos presenting shoots were transferred to Magenta boxes (Magenta Corp., Chicago, IL) containing B+ with half the normal NAA concentration for shoot elongation.

Developed shoots were excised and transferred to Magenta boxes containing RMAN rooting media (Appendix L) (Grosser and Gmitter, 1990) or dipped in 1000 $mg.l^{-1}$ 1-naphthalene-acetic acid (NAA) for 5 min or 3000 $mg.l^{-1}$ indol-butyric acid (IBA) for 3, 5, 7 or 10 min and placed in EMEP with 6% sucrose. NAA and IBA were dissolved with less than 0.3 mL drops of 1 N potassium hydroxide (KOH) + double-distilled water to 100 mL (Smith, 2000 - unpublished data). Rooted plantlets were planted in jiffy pots or plastic pots with commercial mix, covered by plastic bags, and placed in a growth room or directly in the greenhouse.

Shoot grafting. Rooting-recalcitrant shoots were grafted onto sour orange, rough lemon, C-22 or C-146 (*Swingle trifoliate* x Sunki mandarin) rootstock seedlings by cleft grafting, which is usually used for grafting smaller plants such as grapevines or camellias (Kester et al., 1997), in which the rootstock is split and the plant to be grafted is cut on both sides in long wedges to allow efficient cambium-cambium contact. Grafting was held by a transparent soft plastic tube, and the plantlet protected from desiccation by covering it with a small plastic bag. Whole rootstock-plantlet units were covered with plastic and kept in growth room until acclimation in the greenhouse.

Acclimation. Both rooted and grafted plantlets were later transferred to pots containing plant commercial mix and transferred to a the greenhouse for acclimation which was achieved by gardual opening of the plastic covers.

IRRADIATED MICROPROTOPLAST-PROTOPLAST FUSION

Microprotoplast isolation. *S. glutinosa* suspension cells used for microprotoplast isolation had their media changed twice weekly to maintain logarithmic cell growth. Microprotoplasts were isolated following citrus microprotoplast isolation protocol from Louzada et al. (2002). Briefly, 10 mM hidroxyurea (HU) (Sigma-Aldrich Inc, St Louis, MO) were added to early log-phase suspension cells (1 day after sub-culturing) for cell synchronization. After 24 h, cells were washed, three times for 15 min each, with 30 mL of H+H medium, over a horizontal shaker.

Amiprophos-methyl (APM) (Bayer Corporation, Agricultural Division, Kansas City, MO) at 32 µM and 50 mL of H+H medium were added to the flasks to induce micronucleation.

After 24 h, approximately 0.5 g of cells were collected and placed in sterile 0.5 cm Petri dishes containing 2 to 6 mL of BH3 media and 0.5 to 2 mL of enzyme solution, APM 32 µM and 10 µM cytochalasin-B (CB-Sigma, St. Louis), known for decreasing cytoskeletal strength (Thomas et al., 1976), were added. The proportion of media to enzyme solution mixtures was adjusted for the different cultivars.

Cells were digested overnight, in the dark, on rocker platform, with six oscillations per minute.

Protoplasts were filtered through sterile 45 µm mesh stainless steel sieves and washed with 0.6 M BH3 containing 32 µM of APM and 10 µM of CB. Forty µM of CB and 32 µM APM were added to the sucrose 25% and mannitol 13% solutions and sucrose-mannitol gradient was performed as described. Protoplast bands were carefully removed and transferred to clean tubes.

A 7.2% (w/v) mannitol to Percoll™ (Amersham Phamacy Biotech., Piscataway, NJ) solution was placed in 14 x 89 mm centrifuge tubes (Beckman Instruments, Inc., Fullerton, CA) and a iso-osmotic mannitol-percoll gradient was pre-formed by centrifuging it for 30 min at 1000,000 g in a swinging bucket rotor (SW 41 Ti, Beckman Instruments, Inc., Fullerton, CA) to form a mannitol-percoll gradient. Protoplasts were placed on the top of the mannitol-percoll solution and tubes were centrifuged for more two hours at 100,000 g_n at 20 °C.

Bands of microprotoplasts formed were sequentially filtered through nylon sieves of 20, 15, 10 and 5 µm (Small Parts, Inc., Miami Lakes, FL). Small volumes of BH3 media were added to help the filtration process. Filtered fractions were collected in mannitol-BH3 solution, and tubes were centrifuged for 10 min at 80 g_n. Supernatant was re-centrifuged twice for 10 min at 160 g_n and the pellet collected. One to three microprotoplast pellets were isolated; the first containing, probably, the heavier microprotoplasts, the second pellet the lighter microprotoplasts, and the third pellet the lightest.

Pellets were re-suspended with approximately 1 mL of BH3, transferred to microfuge tubes sealed with parafilm and irradiated with doses of 50, 70, 100 or 200 gamma rays (Gy).

Microprotoplast-protoplast fusion. Irradiated *S. glutinosa* microprotoplasts were fused with non irradiated 'Murcott' or 'Ruby Red' protoplasts in a proportion of approximately 3:1, as previously described.

SOMATIC HYBRIDIZATION CONFIRMATION

Hybridity of plantlets from protoplast + protoplast fusion was confirmed by amplified fragment length polymorphism (AFLP) and by flow cytometry analysis. To confirm the presence of the *S. glutinosa* genome in 'Ruby Red' and 'Murcott' tangor, callus derived from microprotoplast + protoplast fusion between *S. glutinosa* and the two species were evaluated by AFLP and dot blot analysis.

DNA extraction. DNA was isolated using DNeasy® Plant Mini kit (Qiagen, Valencia, CA) with minor modifications from callus or suspension cells of parental species, from leaves of plantlets regenerated from the protoplast + protoplast fusion, and from callus produced from the microprotoplast + protoplast fusions.

For calli and suspension cells. Calli and drained suspension cells were ground in liquid nitrogen in a nuclease-free, sterile mortar until a paste was formed. Approximately 100

mg of the paste was put inside sterile 2 mL microfuge tube with 400 μL of buffer AP1 and 8 μL of RNase A stock solution (100 mg.mL^{-1}). Tubes were incubated at 37 ºC over a rocker platform for 30 min plus 10 min at 65 ºC, and tubes were mixed three times during incubation. One hundred and thirty microliters of buffer AP2 were added and incubated on ice for 5 min and centrifuged for 5 min at 20,000 g_n (14,000 rpm). The lysate was applied to a QIAshredder mini spin column in a 2 mL collection tube and centrifuged at 20,000 g_n for 2 min. The flow-through was transferred, without disturbing the cell-debris pellet discarded, to a 2 mL tube, and 1.5 times the volume of the lysate of AP3/E buffer was added and mixed by pippeting. The mix was filtered in a DNeasy mini spin column by centrifuging it for 1 min at 6000 g_n, and the flow-through was discarded. The column was placed in a clean tube, 500 μL of buffer AW was added, and columns were centrifuged for 1 min at 6000 g_n. The flow-through was discarded, an additional 500 μL of buffer AW was added, and the columns centrifuged for 2 min at 20,000 g_n to dry the membrane. The columns were transferred to 1.5 mL microfuge tubes, 50 μL of AE elution buffer were added to the membrane, than incubated at room temperature for 5 min and centrifuged at 6000 g_n for 1 min to elute the DNA. This step was repeated twice. Two microliters of DNA from both elutions were diluted in 98 μL of nuclease free water, and purity and concentrations were measured in a UV/visible spectrophotometer (Amersham Biosciences, Piscataway, NJ). Extracted DNA was stored at -20 ºC.

For leaves. Leaves of 0.5 to 1 cm were collected, frozen in liquid nitrogen and stored at -80 ºC until DNA extraction. Leaves were macerated in liquid nitrogen using a sterile glass stick, 400 µL of buffer AP1 plus 4 µL of RNase A stock solution (100 $mg.mL^{-1}$) were added, and DNA isolated as above.

Dot blotting. DNA was extracted from calli produced from the microprotoplast-protoplast fusion and from the donor and receptor parents.

Target DNA blotting followed the protocol of the Bio-Dot® Microfiltration Apparatus (Bio-Rad, Hercules, CA) and nucleic acid labeling/detection followed the AlkPhos Direct® method (Amersham Biosciences, Piscataway, NJ) with minor modifications, for example, a Zeta-probe® blotting membrane (Bio-Rad, Hercules CA) was used instead of a Hybond-N+ nylon transfer membrane.

For detecting the best target/blocking/probe concentration ratio were performed (Table 2) with the parents DNA in the following combinations:

‘Swinglea’ as target and probe and ‘Murcott’ as blocking;

‘Swinglea’ as target and probe and ‘Ruby Red’ as blocking;

‘Murcott’ as target and blocking and ‘Swinglea’ as probe and;

‘Ruby Red’ as target and blocking and ‘Swinglea’ as probe.

Table 2. Different target/blocking/probe concentration ratios used on a Zeta-Probe membrane. Preliminary studies used genomic DNA from parental species used in the microprotoplast-protoplast fusions.

	30 × blocking DNA			100 × blocking DNA			1000 × blocking DNA		
2 $ng.mL^{-1}$ probe	**0.3 μg target DNA**	**3 μg target DNA**	**9 μg target DNA**	**0.3 μg target DNA**	**3 μg target DNA**	**9 μg target DNA**	**0.3 μg target DNA**	**3 μg target DNA**	**9 μg target DNA**
5 $ng.mL^{-1}$ probe	**0.3 μg target DNA**	**3 μg target DNA**	**9 μg target DNA**	**0.3 μg target DNA**	**3 μg target DNA**	**9 μg target DNA**	**0.3 μg target DNA**	**3 μg target DNA**	**9 μg target DNA**
9 $ng.mL^{-1}$ probe	**0.3 μg target DNA**	**3 μg target DNA**	**9 μg target DNA**	**0.3 μg target DNA**	**3 μg target DNA**	**9 μg target DNA**	**0.3 μg target DNA**	**3 μg target DNA**	**9 μg target DNA**

Denaturation of 0.3, 3 and 9 $\mu g.\mu L^{-1}$ of target DNA ('Ruby Red', 'Murcott' or 'Swinglea') was accomplished by addition of 0.4 M sodium hydroxide (NaOH) solution and 10 mM ethylenediaminetetraacetic acid (EDTA) solution. Samples were heated to 100 ºC for 10 min and neutralized by adding equal volume of cold 2 M ammonium acetate pH 7.0. The membrane was pre-wet for 10 min in distilled water before placing inside the Bio-Dot apparatus. Membranes were re-hydrated, under vacuum, with 150 µL of water per well, before applying the samples. After samples passed through the membrane, 500 µL of 0.4 M NaOH were applied to each well. The membrane was removed from the apparatus, rinsed with 2x SSC (sodium chloride – sodium citrate buffer), and allowed to air dry. The membrane was cut in pieces representing the different treatments and put inside 5 cm diameter Petri dishes. The squared area of the blots was measured, and 0.25 $mL.cm^2$ of pre-warmed (55 ºC) hybridization buffer [0.5 M of sodium chloride (NaCl) and 4% (w/v) of AlkPhos Direct® blocking reagent (Amersham Biosciences, Little Chalfont Bickinghamshire, UK)] containing 30×, 100×

or 1000× blocking DNA were poured onto the blots. The blocking DNA was physically broken by passing through a syringe with needle, heated at 100 ºC, and placed on ice for 5 min before its addition to the hybridization buffer. Pre-hybridization was performed for 15 min at 55 ºC in Shake 'N' Bake^{TM} hybridization oven (Boekel Scientific, Feasterville, PA) under gentle agitation (60 strokes per min). The DNA used as a probe was diluted to 10 ng.µL^{-1} and 2, 5 and 10 ng of AlkPhos Direct® chemiluminescent (Amersham Biosciences, Piscataway, NJ) labeled probe DNA per mL of buffer were added to the blots and allowed to hybridize overnight inside the hybridization oven at 55 ºC under gentle agitation.

Blots were washed twice with 2 mL/cm^2 primary wash buffer [2 M urea, 0.1% (w/v) sodium dodecyl sulfate (SDS), 50 mM sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$), 150 mM of NaCl, 1mM of magnesium chloride ($MgCl_2$) and 0.2% (w/v) of blocking reagent] at 55 ºC for 10 min with agitation, transferred to clean container and washed twice with 2 mL/cm^2 secondary buffer [1 M Tris base, 2 M NaCl] at room temperature for 5 min under agitation. Excess buffer was drained, and blots were placed over a plastic covered cardboard and covered with 35 µL/cm^2 of CPD-*Star*^{TM} chemiluminescence (Amersham Biosciences, Piscataway, NJ) detection solution for 5 min. Excess detection reagent was drained. The DNA blots were placed DNA side up in a film cassette and exposed for 1 to 3 h to Biomax^{TM} MS autoradiography film (Kodak, Rochester NY), in the dark, at room temperature, and processed manually by immersing films in Kodak GBX developer and replenisher (Eastman Kodak Company, Rochester, NY) for 5 min; transferring to Kodak Indicator Stop Bath (Eastman Kodak Company, Rochester, NY) for 30 sec, under

constant agitation; immersing in Kodak GBX fixer and replenisher (Eastman Kodak Company, Rochester, NY) for 7 min; washing under running water for 5 min and allowing them to dry at room temperature.

A dot blot analysis using DNA extracted from callus produced by the fusions of irradiated ‘Swinglea’ microprotoplasts with non irradiated ‘Ruby Red’ protoplasts, named as H2, H3, H4, H5, H6 and H7 was performed. Three micrograms of H2, H3, H4, H5, H6 and H7 were used as target DNA, 1000 × ‘Ruby Red’ DNA was used as the block and 5 ng of ‘Swinglea’ were used as the labeled probe.

Two additional dot blots were performed, using DNA extracted from callus produced by fusion of irradiated ‘Swinglea’ microprotoplasts with non irradiated ‘Murcott’ protoplasts, named H8. Three micrograms of H8 were used as target DNA. For blocking, 1000 × ‘Murcott’ DNA was used in one of the dot blots and 1000 × H8 DNA in the other. Five nanograms of ‘Swinglea’ DNA were used as labeled probes in both experiments.

Amplified fragment length polymorphism (AFLP) analysis. AFLP analysis was performed in a 4300 DNA analyzer (Li-Cor, Inc. Lincoln, NE) using the IRDye® Fluorescent AFLP® Kit (Li-Cor® Biosciences, Lincoln, NE) with some adjustments. Briefly: for restriction digestion of genomic DNA, 100 ng of template DNA in less than 9 µL were used, plus 1 µL of *Eco*RI/*Mse*I enzyme mix [1.25 units/µL each in 10 mM Tris-HCl (pH 7,4), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 200 $\mu g.mL^{-}1$ BSA 50% (v/v) glycerol, 0.15% Triton X-100], 2.5 µL 5X reaction buffer [50 mM Tris-HCl (pH

7.5), 50 mM magnesium-acetate, 250 mM potassium-acetate] were combined and deionized water was added to 12.5 μL total volume and incubated at 37 ºC for 2 h. The enzyme was inactivated at 70 ºC for 15 min and placed on ice to inactivate the restriction enzymes. Adapter ligation was performed by adding to the previous solution 12 μL of adaptor mix [*EcoRI*/*Mse*I adapters, 0.4 mM ATP, 10 mM Tris-HCl (pH 7.5) 10 mM magnesium-acetate, 50 mM potassium-acetate], 0.5 μL of T4 DNA ligase and incubating the mixture at 20 ºC for 2 h. Ten microliters of the mixture were diluted 1:10 by adding 90 μL of TE buffer [10 mM Tris-HCl (pH 8.0), 1.0 mM EDTA]. Pre-amplification was performed by adding 2.5 μL of the 1:10 diluted ligation mixture to a 0.2-mL PCR tube containing 20 μL of AFLP® Pre-amp primer mix, 2.5 μL of 10× PCR reaction buffer [100 mM Tris-HCl (ph 8.3), 15 mM $MgCl_2$, 500 mM KCl], and 0.5 μL Taq DNA polymerase (2.5 units/μL) (Roche Molecular Biochemicals, Indianapolis, IN); Thirty cycles at 94 ºC for 30 sec, 56 ºC for 1 min and 72 ºC for 1 min were performed. *Mse* primers used for selective amplification were from MWG (MWG Biotech AG, Ebersberg, Germany) and Operon (Operon Biotechnologies, Inc., Huntsville, AL). For selective amplification, 2 μL of pre-amplified DNA, 1.96 μL of nuclease free water, 1 μL of 10 × buffer (Promega Corporation, Madison, WI), 1 μL of 25 mM MgCl, 1 μL of 2 mM dNTPs, 0.04 μL of Taq polymerase (5 units/μL), 2 μL of *Mse*I primer and 0.5 μL of both 700 and 800 IRDye *EcoRI* primer were used. One cycle of 94 ºC for 30 sec, 65 ºC for 30 sec, and 72 ºC for 1 min; twelve cycles of 94 ºC for 30 sec, 65 ºC for 30 sec, and 72 ºC for 1 min; and 23 cycles of 94 ºC for 30 sec, 65 ºC for 30 sec, and 72 ºC for 1 min were performed. After amplification 2 μL of the samples were diluted with 8 μL of

nuclease-free water and 5 µL of dye (Li-Cor® Biosciences, Lincoln, NE). Samples and ladder (Li-Cor® Biosciences, Lincoln, NE) were denatured for 3 min at 94 ºC and placed on ice. Each sample (0.5 µL) was loaded on a 96-well polyacrylamide gel and image data was viewed and printed using Saga^{GT} software.

*Eco*RI labeled primers contained in the Licor kit and other unlabeled *Mse*I primers were tested in different primer combinations (Tables 3, 4 and 5).

Flow cytometry. Ploidy analysis was performed by flow cytometry**.** Leaves (0.5 to 1 cm) from the protoplast-protoplast fusion plants were collected and shipped on ice to the Citrus Research and Education Center, University of Florida, Lake Alfred, were flow cytometry analysis was kindly performed by Dr. Jude Grosser on a Partec ploidy analysis machine (D-48161, Münster, Germany) following the method reported by Miranda et al. (1997).

Table 3. Primer combinations tested to identify microprotoplast-protoplast hybrids.

***Mse*I unlabeled primers**	***Eco*R1 labeled primers**
CAG	ACC
	AGG
	CAG
CAA	ACA
	AGG
	CAA
CA	ACA
	AGG
	AAC
	ACT
	AAG
	ACC
CT	ACT
	ACA
	ACG
	AAC
	AGC
	AAG
	AGG
	ACC
	CAT

Table 4. Primer combinations tested to observe polymorphisms in the parents.

***Mse*I unlabeled primers**	***Eco*R1 labeled primers**
CA	ACA
	AGG
	AAC
	ACT
	AGG
	ACC
CT	AGG
	AAG
	ACG
	AAC
	ACC
	ACT
	ACA
AC	AGC
	AAG
TC	ACG
	AAC

Table 5. Primer combinations tested to identify protoplast-protoplast hybrids.

***Mse*I unlabeled primers**	***Ec*oR1 labeled primers**
CAA	ACA
	AGG
CT	ACT
	ACA
	ACG
CA	AGG
	ACC
	ACT
	ACA

RESULTS

PROTOPLAST ISOLATION IN THE DIFFERENT CULTIVARS

It was possible to isolate protoplasts from most of the cultivars tested. However, cultivars responded differently to the isolation protocol, and individual adjustments had to be performed for maximization of good quality protoplasts. Quality was defined by visual appearance under a microscope, i.e. protoplasts were round, completely free of cell wall or easily detachable from cell wall debris, and did not burst during enzymatic digestion and manipulation.

ENZYME SOLUTION: MEDIUM PROPORTION USED FOR PROTOPLASTS ISOLATION IN THE DIFFERENT CULTIVARS

Proportions of enzyme solution to BH3 medium had to be individually adjusted for each cultivar.

A 2:1 medium:enzyme solution proportion was tested with cells of ‘Hanlim’, ‘Itaborai’, Valencia’, ‘Flame’ and ‘Ruby Red’, however, only very thin protoplast band of ‘Ruby Red’, ‘Itaborai’ and ‘Flame’ were formed during the sucrose-mannitol gradient and the amount of protoplast was insufficient for isolation. Higher proportions of enzyme allowed digestion in less time, i.e. 8. However, cell clumps were observed with their core protected from the enzymes, while the external cells were digested fast and usually burst. Hence, a suitable amount of protoplasts for isolation was not formed because protoplasts were being formed gradually, and before the enzyme reached the

core of the cell clumps, the previously formed would be vulnerable to overdigestion by the high concentration of enzyme and would usually burst.

When a 3:1 medium: enzyme solution proportion was tested with cells of ‘Hanlim’, ‘Succari’, ‘Natal’, ‘Flame’ and ‘Ruby Red’; ‘Flame’ and ‘Natal’, it took approximately ten hours to form enough protoplasts for isolation. ‘Succari’ took more than 11 hours and ‘Ruby Red’ almost 17 h. ‘Hanlim’ protoplasts could not be isolated, and only clumps of undigested cells and detached over-digested cells were observed.

When “Ruby Red’, ‘Flame’’ ‘Natal’ and ‘Succari’ were digested in a 3:0.5 medium:enzyme solution, a good amount of ‘Flame’ protoplasts were obtained within 17 h. A reasonable amount of ‘Natal’ protoplasts were formed. However, during isolation, they would not float in 25% sucrose, so 35% sucrose was used. The 3:0.5 medium:enzyme solution proportion did not work for ‘Succari’. Many ‘Ruby Red’ protoplasts were obtained, but their formation took 21 h of incubation, i.e., much longer than for ‘Flame’.

When ‘Valencia’, ‘Itaborai’, ‘Natal’, ‘Flame’ and ‘Succari’ were incubated in a 4:0.5 medium:enzyme solution, cell walls were digested, 18 h were necessary to obtain adequate protoplast formation. However, it seemed that prolonged digestion in lower medium:enzyme solution proportion was more gentle and resulted in larger number of viable protoplasts. The exception was the ‘Valencia’ cultivar, which did not form protoplasts.

For the cultivar Hanlim, protoplasts formation was not successful, even using low medium:enzyme solution proportions such as 4:0.5 and 5:0.5.

A 4:0.5 medium:enzyme solution proportion was tested with cells of 'Satsuma', 'Murcott' and 'Changsha'. Some 'Satsuma' protoplasts were isolated after 16 h digestion. 'Murcott' presented cell clumps and a few released protoplasts which burst. 'Changsha' cells did not digest well, even with different medium:enzyme solution proportions (2:1 and 2:2). By changing the molarity of the BH3 medium and enzyme solutions from 0.6 (used for all the other cultivars) to 0.7, and using the ratio 3:0.5 medium:enzyme solution, a large number of 'Murcott' and 'Changsha' protoplasts was obtained after 16 h incubation.

A gradual digestion was tested with some of the cultivars which were presenting problems. Cell from the cultivars 'Itaborai' and 'Succari' were incubated in 2:0.5 BH3 medium:1% macerozyme solution on a shaker at 6 rpm, for 2 h. Then, 2:0.5 of BH3 medium:1% cellulose and 0.2% pectolyase enzyme solution were added to the plates. For the 'Valencia' cultivar, 2:0.25 was added to pre-digest the cells, and two hours later, 3:0.25 was added. This gradual digestion was very efficient for the above problematic cultivars and good number of protoplasts was isolated. However, for 'Hanlim', no proportion was efficient in isolating protoplasts, even when using gradual digestion.

Most cultivars yielded an adequate number of protoplasts (Fig. 1). The cultivars which produced fewer protoplasts were "Valencia' and 'Natal', in which high content of starch could be observed. Their protoplasts broke easily, and protoplast bands were not easily formed since protoplasts usually stayed in the bottom of the flask.

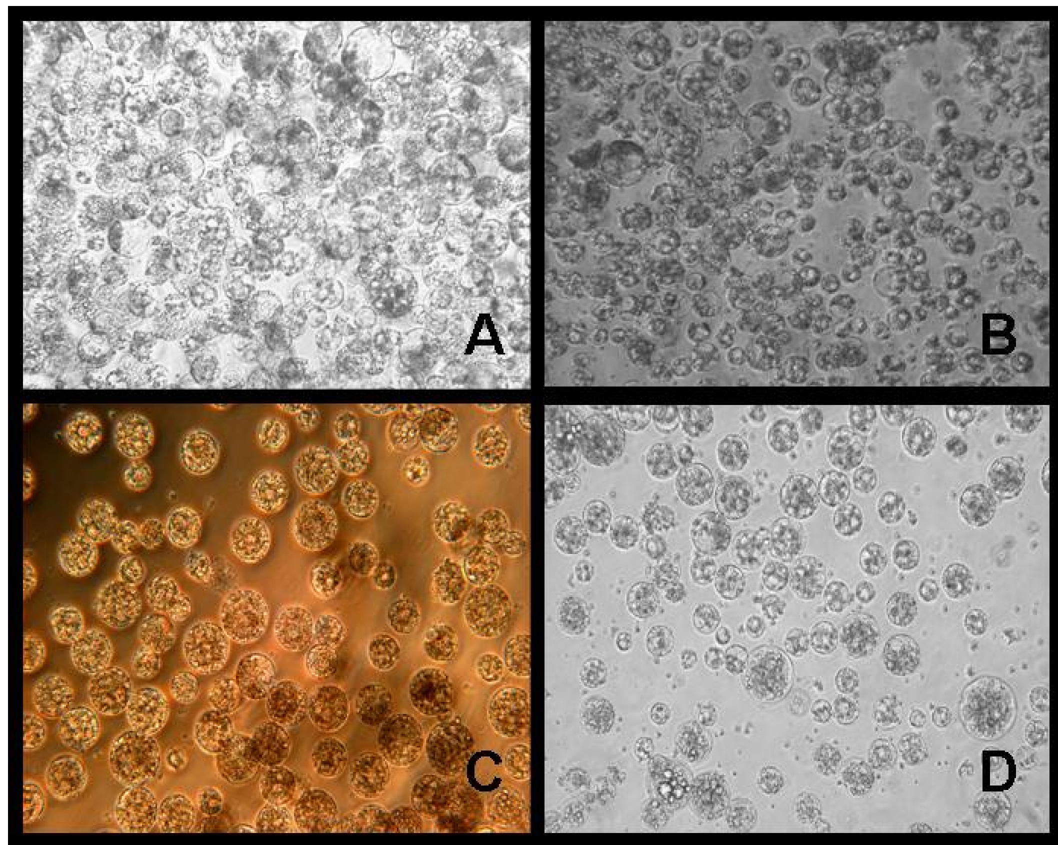

Fig. 1 Ruby Red' protoplasts (A); 'Itaborai' protoplasts (B); 'Flame' protoplasts (C); 'Succari' protoplasts (D).

Briefly, the following enzyme medium:solution proportions were chosen: 3:1 for 'Ruby Red'; 3.0.5 for 'Murcott'; 4:0.5 for 'Flame', 'Natal' and 'Satsuma', and gradual digestion 2:0.5 + 2:0.5 for 'Itaborai' and 'Succari' and 2:0.25 + 3:0.25 for 'Valencia'.

Based on their protoplast isolation capacity, the cultivars 'Ruby Red', 'Flame', 'Itaborai', 'Natal', Valencia', 'Succari', 'Satsuma' and 'Murcott' were chosen for further experiments. The cultivars 'Hanlim' and 'Changsha' did not form viable protoplasts or did not produce enough quantities.

PROTOPLAST FUSION

Approximately equal numbers of protoplasts were fused in different combinations, preferentially grapefruit with sweet orange, or 'Murcott' tangor with a sweet orange.

Fusions of two, three or more protoplasts were observed under an inverted microscope (Fig. 2).

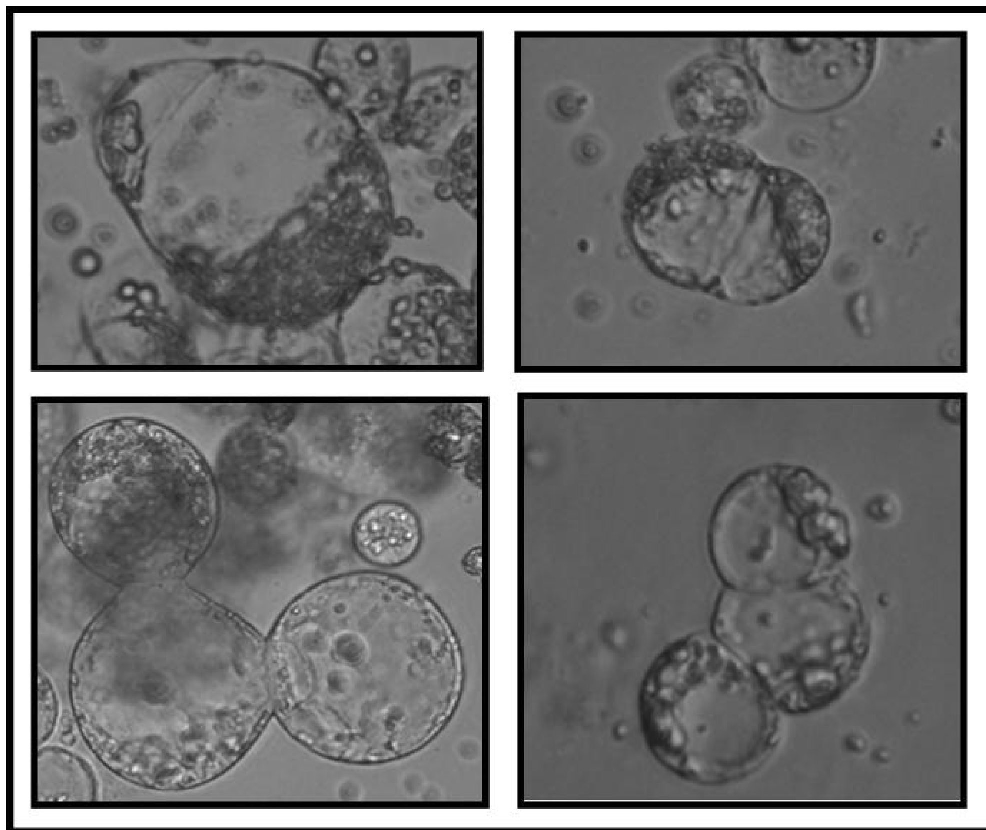

Fig. 2. Protoplast fusions showing two and three protoplasts fusing.

PROTOPLAST CULTIVATION

Fused protoplasts were cultivated in the dark for the first 30 days. Cell wall formation was observed in approximately 3 to 5 days of culture after the protoplasts-protoplasts normal fusions (P-P), and from one to two weeks for those treated with irradiation plus IOA. Following P-P, the cells started to enlarge after approximately 8 to 10 days in preparation for division and a few cell divisions were observed (Fig. 3). Masses of calli from P-P were observed within 3-4 weeks (Fig. 4) whereas their appearance from the protoplasts-irradiated protoplasts (P-I) and IOA treated protoplasts-irradiated protoplasts (IOA-I) took 6-8 weeks.

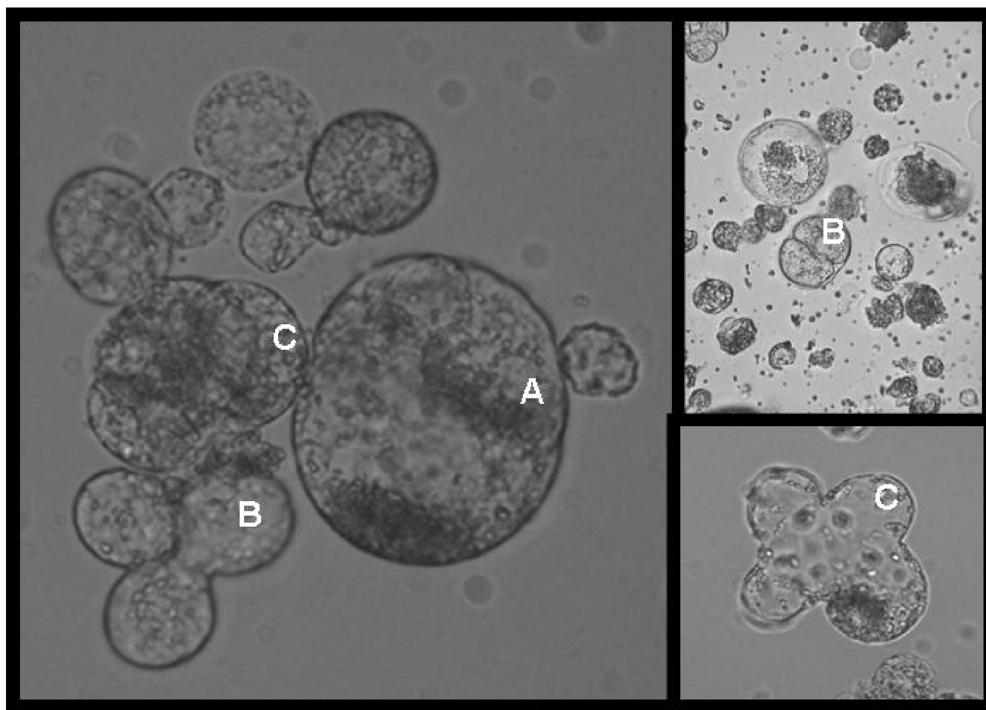

Fig. 3. Cell before division (A); Cell dividing (B); Microcralli forming (C).

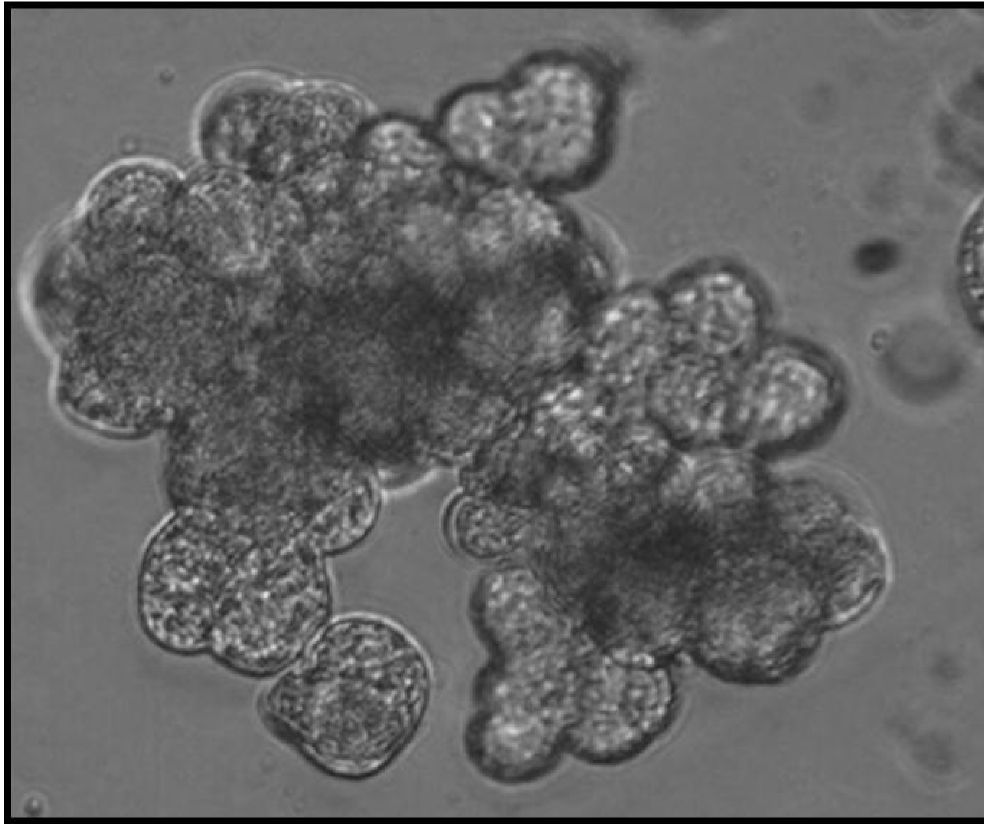

Fig. 4. Microcalli produced from protoplast fusion.

P-P calli was transferred to solid EMEP within approximately one and a half months and within two to two and a half months to P-I and IOA-I fusions. Larger calli were easier to transfer as could be handled with tweezers whereas smaller ones had to be pippeted with liquid BH3. Additionally, less liquid BH3 was transferred using tweezers, so less contamination occurred and embryos appeared earlier. After embryo formation, no significant developmental differences were observed among P-P, P-I and IOA-I fusions. However, no embryo was formed on callus from protoplasts irradiated at 150 Gy or higher.

As soon as embryos were formed they were transferred to fresh solid EMEP. Embryos that were not transferred returned to callus stage. Sub-culturing in EMEP was performed about once monthly, depending of embryo growth. Embryos took around three sub-cultures to reach about 0.5 cm long at which they were transferred to 1500 medium for further development. After a few months, it was noticed that this was an unnecessary step and so, embryos were allowed to stay longer on solid EMEP before being transferred to B+ medium to induce embryo elongation.

Poorly developed embryos were transferred to DBA3, which is a medium rich in cytokines to induce shoot formation. However, embryos became deformed in DBA3 medium so its use was terminated. Sub-culturing in B+ was performed as necessary until shoots started to develop. Shoot formation started after approximately 1 year from fusion.

Shoots were cut and placed in magenta boxes containing RMAN rooting media for rooting induction. Many shoots were lost during this phase of *in vitro* cultivation because they did not root and started to turn yellow and die. Only one shoot from the 'Ruby Red' + 'Succari' fusion rooted (Fig. 5). However, the plant died during acclimation after 1.5 months in the greenhouse.

Fig. 5. Spontaneously rooted shoot formed from a ‘Ruby Red’ + ‘Succari’ protoplast fusion.

Some shoots were grafted onto rough lemon, C-22 or C-146 rootstocks, covered with plastic, and kept inside growth room until the graft was well bonded and shoots were feeding from the rootstocks (Fig. 6). Around 50% mortality was observed during this process. Some of the grafted shoots dehydrated, therefore, smaller plastic covers were used. However, shoots were attacked by fungi, probably because of the higher humidity environment created inside the plastic. Some shoots died because the graft did not bond well or from bacteria infections, probably due to tool manipulation during

grafting. Grafting onto C-146 was inferior in that took longer to seal the graft during which some shoots died for lack of nourishment.

Fig. 6. Shoot grafted onto rootstock, held by a malleable plastic tube and covered with a plastic bag.

To induce strong shoot formation for later rooting attempts, embryos with developing shoots were transferred to magenta boxes containing B+ with half the amount of NAA. This enabled shoot elongation (Fig. 7). It seems that once the auxin content was reduced to half and physical space was provided for elongation, shoots formed easily.

Fig. 7. Embryo germination and plantlets regenerated.

To solve the recalcitrance to rooting, shoots were dipped in 1000 mg l^{-1} NAA for 5 min or in 3000 mg l^{-1} of IBA for 3, 5, 7 or 10 min (Fig. 8A) and placed in magenta boxes containing EMEP with 6% sucrose (Fig. 8B). Rooting occurred after shoots were dipped in IBA for 10 min (Fig. 9).

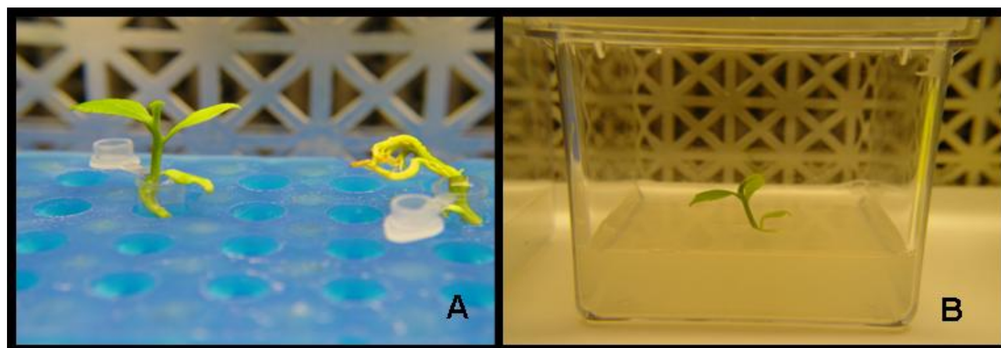

Fig. 8. Shoots dipped in 3000 mg.l^{-1} IBA solution (A). Shoot placed in magenta box containing EMEP with 6% sucrose for rooting (B).

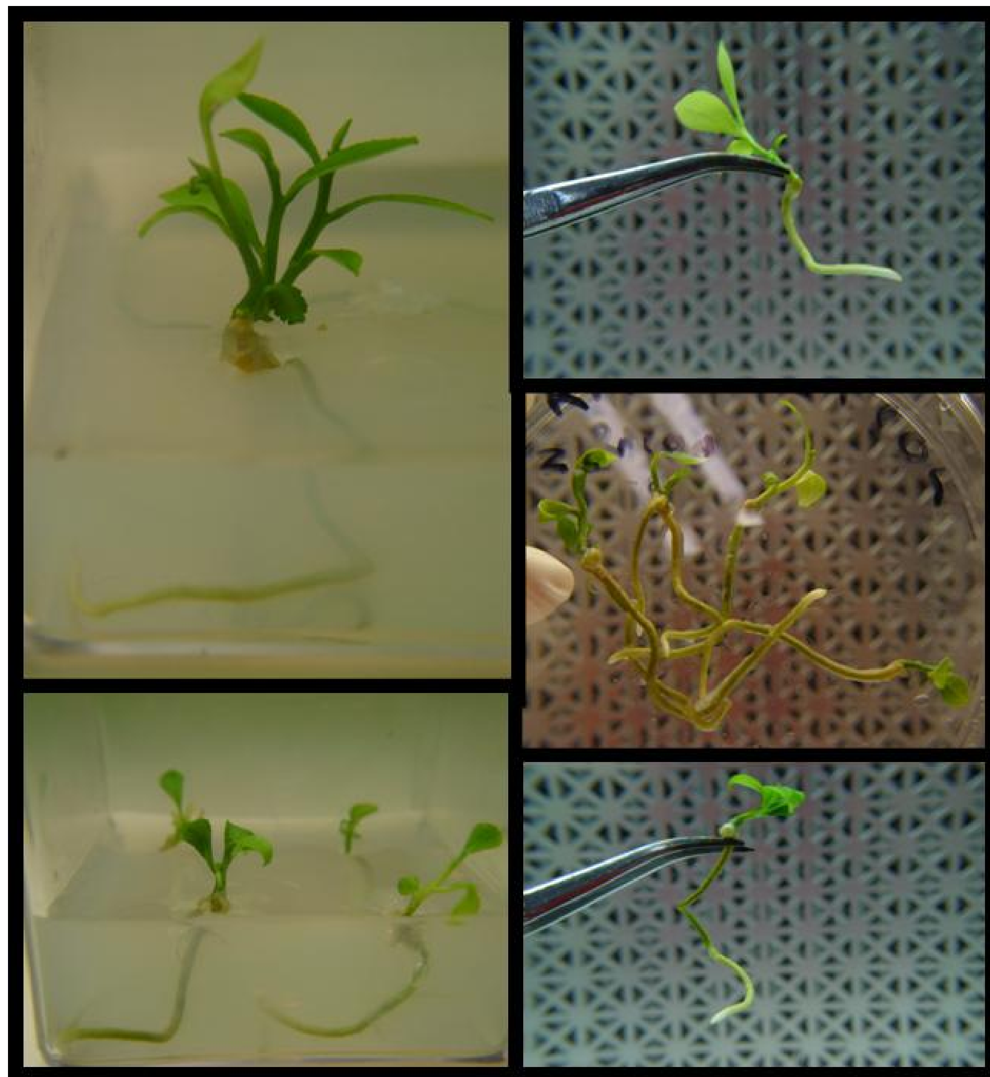

Fig. 9. Rooted shoots after dipping in 3000 mg l^{-1} IBA for 10 min and placed on EMEP with 6% sucrose.

The basic EMEP medium with extra sucrose accommodated the rooting plants very well, probably due to both the extra energy provided by the sucrose and the lack of hormonal conflict.

Once the successfully grafted plants were acclimated, their development was fast (Fig. 10B). Development of plants over their own roots was better when shoots were

allowed to grow larger (> 2 cm) *in vitro* before dipping in IBA for rooting. Bigger plants developed faster in soil than the small ones (Fig. 10A).

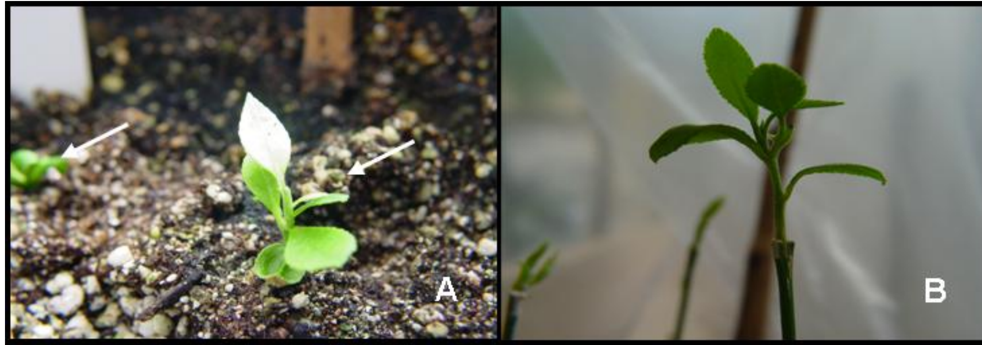

Fig. 10. 'Ruby Red' 20' IOA + 'Succari' 80 Gy tetraploid plant (A).'Ruby Red' 20' IOA + 'Succari' 80 Gy tetraploid shoot grafted onto C-22 rootstock (B).

SYMMETRIC HYBRIDIZATION

Eight shoots, all from 'Ruby Red' + 'Itaborai' fusion, were grafted and transplanted to the greenhouse. Only two shoots survived acclimation. They resembled 'Itaborai' and grew vigorously (Fig. 11). Both plants were diploid, as shown by the flow cytometry analysis (Fig. 12).

Fifteen shoots from the 'Ruby Red' + 'Itaborai' fusion were dipped in 3000 $mg.l^{-1}$ IBA, formed roots, and were planted in jiffy pots. After approximately two weeks, plants were transplanted to pots containing commercial potting mix in the greenhouse. Seven plants survived the acclimation process. Some plants were lost to fungal infection due to high humidity in the plastic bags.

Fig. 11. Diploid ‘Ruby Red’ + ‘Itaborai’ shoot grafted onto rough lemon.

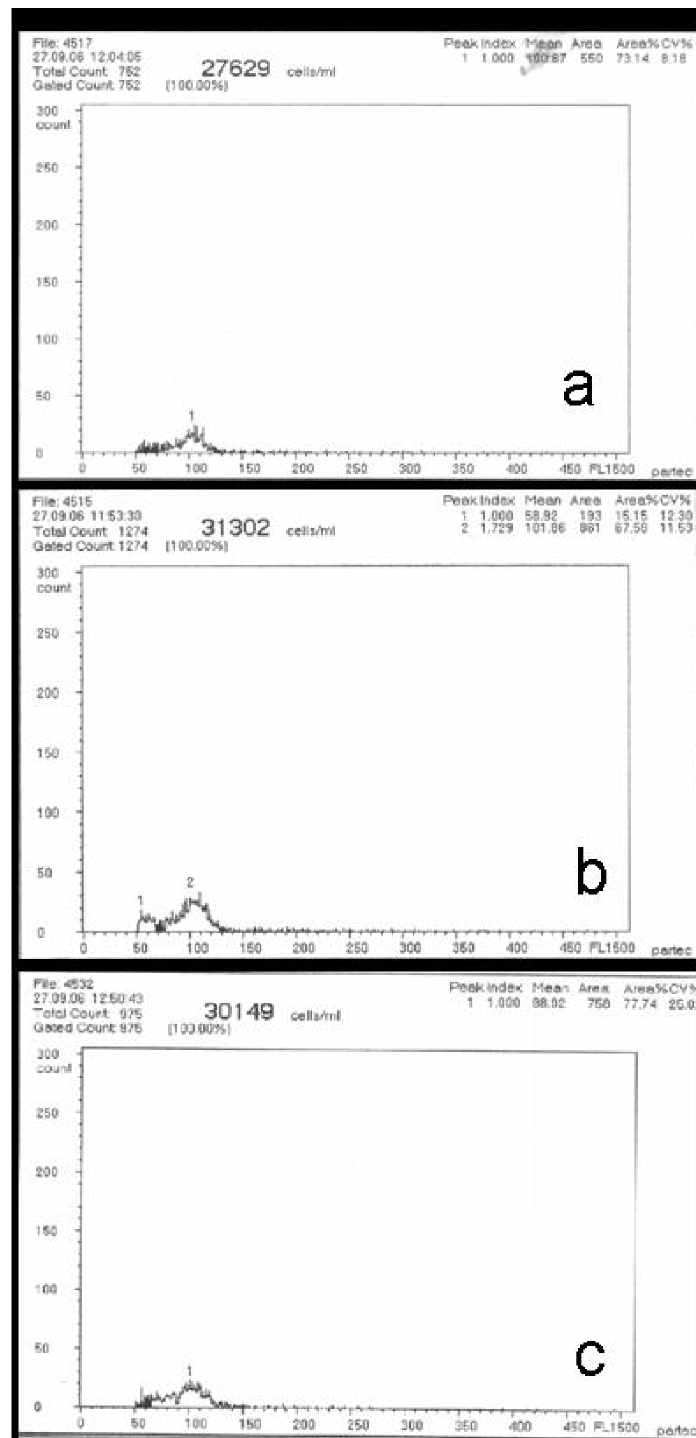

Fig. 12. Flow cytometry diagnosis. Diploid standard (A). Diploid ‘Ruby Red’ + ‘Itaborai’ plant (B). Diploid ‘Ruby Red’ + ‘Itaborai’ plant (C).

ASYMMETRIC HYBRIDIZATION

Irradiation tolerance was different among varieties. However, no one survived 150 and up gamma rays exposure.

The fusions involving irradiated 'Satsuma' protoplasts did not form embryos and fusions involving 'Flame' protoplasts did not developed further then the embryo stage.

Fusions of 'Ruby Red' + 'Itaborai' irradiated, 'Ruby Red' + Succari' irradiated and 'Itaborai' + 'Ruby Red' irradiated produced some shoots and are still producing. However, the first formed shoots were almost all lost during the media optimization, grafting, rooting experimentation and acclimation.

There is one shoot of 'Ruby Red' + 'Itaborai' irradiated at 100 Gy grafted onto Rough lemon in greenhouse which present a peculiar ploidy; between triploid and tetraploid (Figure 13). However, it presents slow growth.

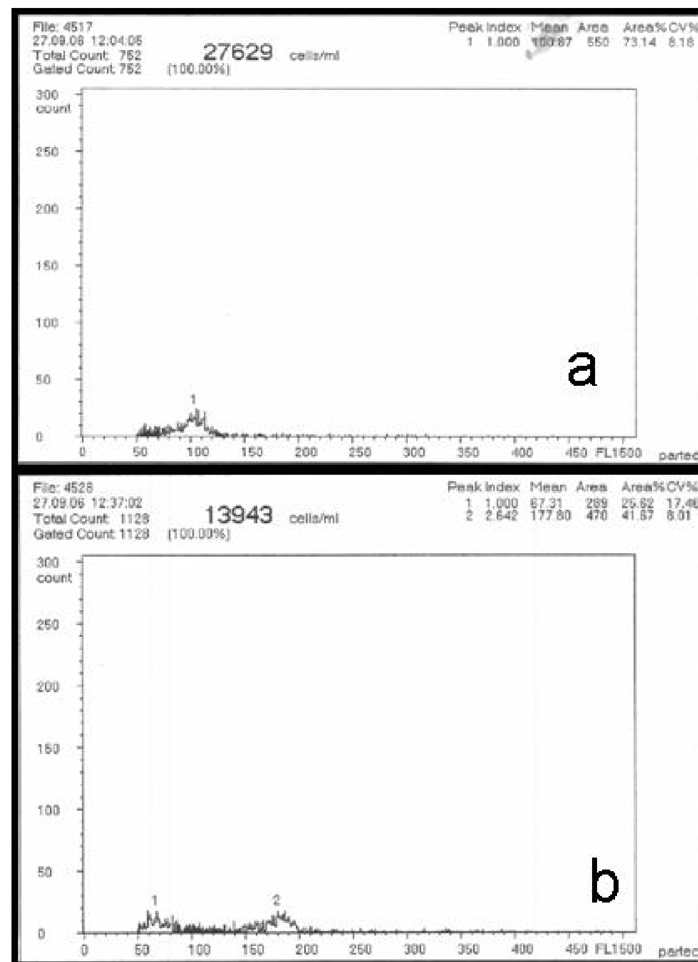

Fig. 13. Flow cytometry diagnosis. Diploid standard (A). Aneuploid 'Ruby Red' + 'Itaborai'100 Gy plant (B).

Four shoots from the 'Itaborai' 100 Gy controls were obtained. Two grafted and two rooted. The rooted plants were small and weak and both died. One of the grafted shoots was small and chlorotic and died while the other one was normal and was classified as diploid by flow cytometry.

IOA plus irradiation for double inactivation. A 3 mM IOA treatment precluded further development of the protoplasts independently of exposure time (10, 13, 15 or 20 min). Treated cells did not divide and degenerated (Fig. 14).

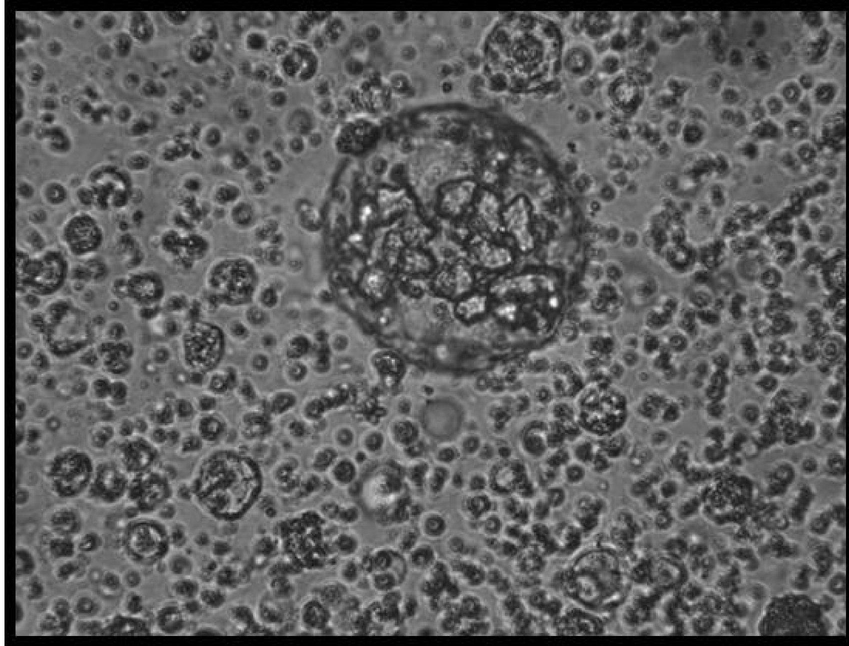

Fig. 14. IOA treated 'Ruby Red' protoplasts could not divide and disintegrated

Cell division and multiplication were different among the IOA-treated, irradiated and fusion cells (Fig. 15). IOA treated cells could not divide; division in irradiated cells was delayed; fused protoplasts divided and multiplied, forming microcalli, probably due to complementation.

Fig. 15. IOA treated protoplasts; Irradiated protoplasts; fused protoplasts (Top to bottom).

The fusion of 'Itaborai' protoplasts exposed to 3 mM IOA for 15 min with 'Murcott' protoplasts exposed to 50 Gy produced two shoots. One rooted but did not survive acclimation, and the other was grafted onto C-22. However, its leaves did not expand well.

IOA-20 min 'Murcott' plus 100 Gy 'Itaborai' or 'Natal' fusions produced two plants, one from each treatment. However, they did not survive the acclimation process.

Fusions involving 'Changsha' did not form embryos.

A plant was obtained from the IOA-15 min 'Ruby Red' plus 100 Gy 'Itaborai' fusion and another one from the IOA-20 min 'Ruby Red' plus 100 Gy 'Itaborai' fusion. Their morphologies were very distinctive from the plants produced by double inactivation involving 'Succari' (IOA treated 'Ruby Red' protoplasts + irradiated 'Succari' protoplasts). However, no further analysis was performed because they were too small to collect samples for DNA extraction or flow cytometry.

Some shoots from fusions of the 20 min IOA treated 'Ruby Red' protoplasts + 100 Gy 'Succari' irradiated protoplast fusion were still forming in ½ NAA B+ media and others were already in the rooting process.

One shoot was produced from the 15 min IOA treated 'Ruby Red' protoplasts + 30 Gy irradiated 'Succari' protoplasts fusion, and it was grafted onto C-22. Shoots from this treatment continued production in ½ NAA B+ medium.

The best combination, which led to many plants, was the IOA-20 min 'Ruby Red' plus 80 Gy 'Succari' fusion. This treatment has already yielded 37 plants and many shoots continue to form in ½ NAA B+, and two shoots continue to root in EMEP 6%

(Fig. 16). During acclimation, eight plants from this treatment were lost. Samples from 13 plants from this fusion combination were sent for flow cytometry analysis and all were found to be tetraploid (Fig. 17). IOA-20 min 'Ruby Red' or 80 Gy 'Succari' protoplasts alone were cultured as control but did not develop.

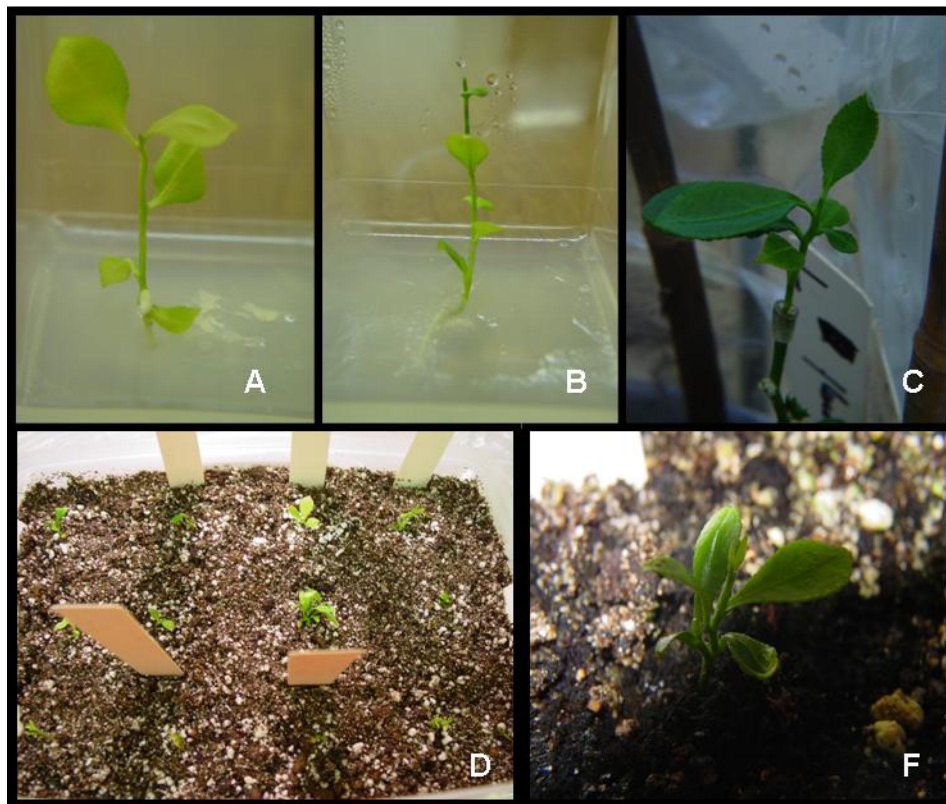

Fig. 16. Plants from the 20 min 'Ruby Red' + 80 Gy irradiated protoplast fusion. *In vitro* (A and B); Grafted (C); Planted in commercial soil mix (D and F).

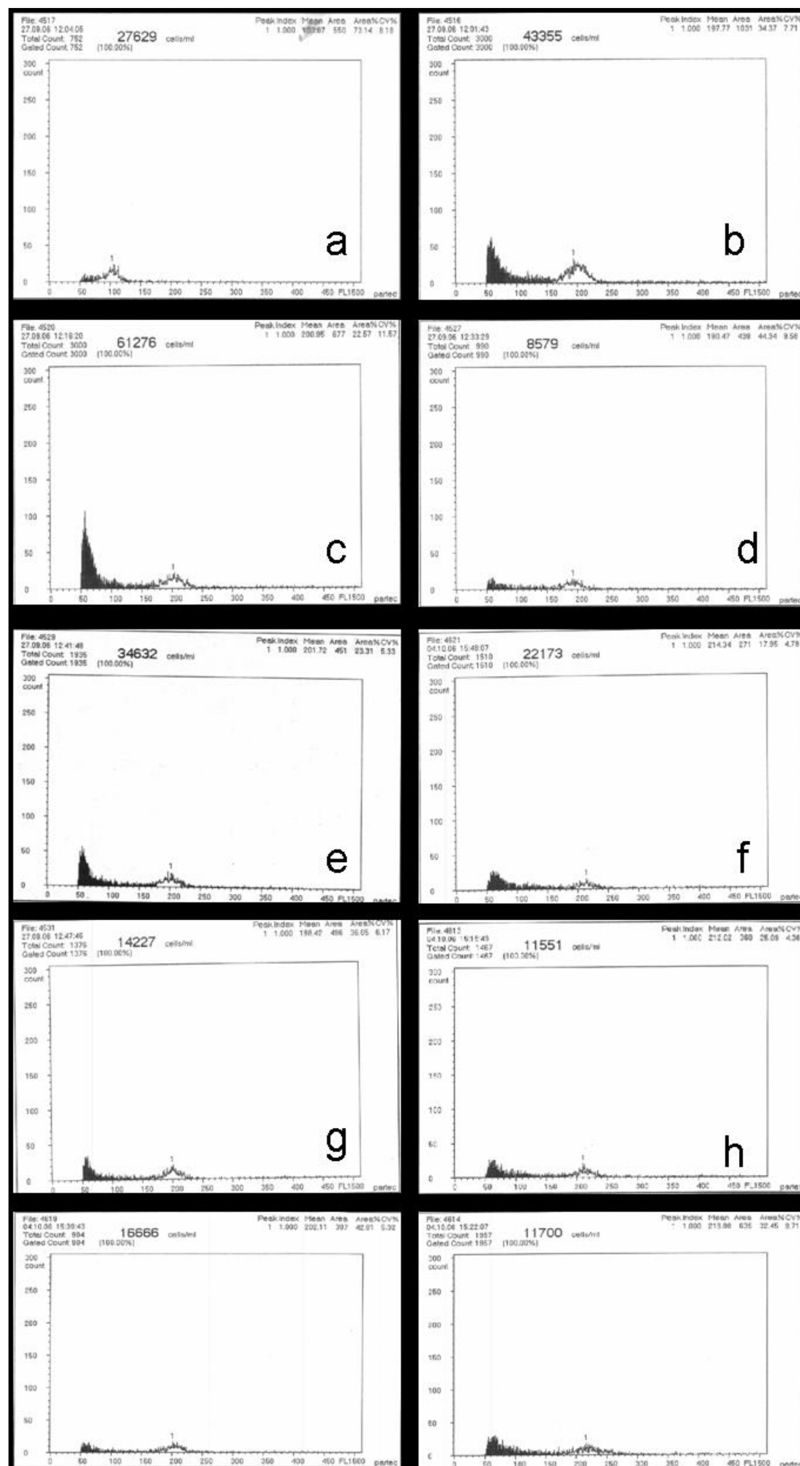

Fig. 17. Flow cytometry diagnosis. Diploid standard (A). Tetraploid IOA-treated ‘Ruby Red’ + 80 Gy irradiated ‘Succari’ plants (B-G).

AFLPs. Good banding patterns were observed for most primer combinations, and some revealed polymorphisms. The technique was straightforward and is highly reproducible.

'Murcott' + sweet orange protoplast fusions. One shoot was produced from the IOA-15 min 'Itaborai' + 50 Gy 'Murcott' (Z5). Its morphology was different from both 'Ruby Red' + 'Succari' and 'Ruby Red' from 'Murcott' fusions. Shoot elongates slowly and leaves are small and not well developed.

One shoot was produced from the 20 min IOA treated 'Murcott' + 100 Gy irradiated 'Natal' (Z10). However, the shoot was lost to fungal contamination.

AFLP analysis from both shoots showed bands from both parents with *Mse*I-CT plus *Eco*RI-ACT, *Mse*I-AC plus *Eco*RI-ACT, and *Mse*I-CAA plus *Eco*RI-AGG primer combinations (Fig. 18).

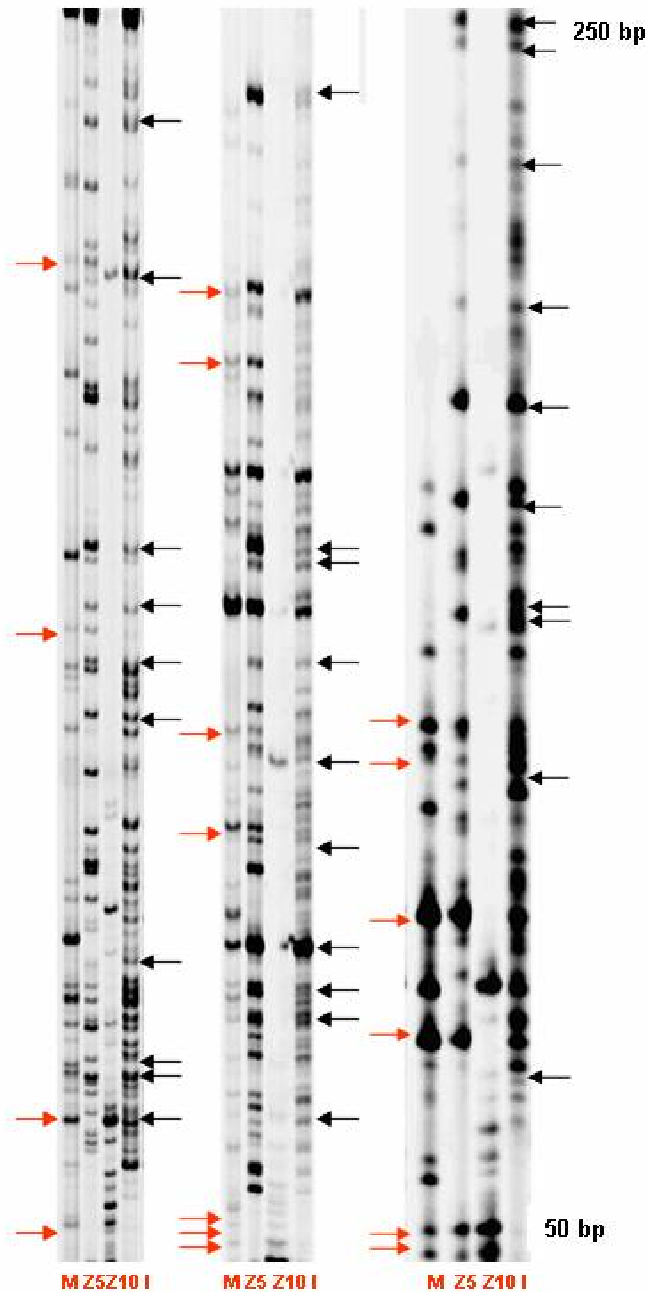

Fig. 18. 'Murcott' (M) and 'Itaborai' (I) parents and complementary parental band morphology of Z5 (15' IOA 'Itaborai' + 50 Gy 'Murcott') and Z10 (20' IOA 'Murcott' + 100 Gy 'Natal') with *Mse*I-CT plus *Eco*RI-ACT, *Mse*I-AC plus *Eco*RI-ACT and *Mse*I-CAA plus *Eco*RI-AGG primer combinations (left to right panel).

‘Ruby Red’ + sweet orange irradiated protoplasts. ‘Ruby Red’ and sweet oranges presented very similar AFLP patterns. The DNA samples from many ‘Ruby Red’ + irradiated ‘Itaborai’ and ‘Ruby Red’ + irradiated ‘Succari’ shoots were analyzed. Polymorphisms were observed with different primer combinations.

The *Mse*I-CAA plus *Eco*RI-ACA primer combinations revealed at least nine hybrid samples. Five of them from the 20 min IOA “Ruby Red’ + 80 Gy ‘Succari’ fusion, two from the ‘Ruby Red’ + ‘Itaborai’ fusion, one from the 15min IOA ‘Ruby Red’ + 30 Gy ‘Succari’ fusion, and one from the peculiar ploidy (between triploid and tetraploid) ‘Ruby Red’ + 100 Gy ‘Itaborai’ fusion (Fig. 19). This same plant (F) was also shown to be hybrid with two other primer combinations, *Mse*I-CT plus *Eco*RI-ACA and *Mse*I-CT plus *Eco*RI-ACT. These two primer combinations also showed band polymorphism in many samples (Fig. 20 and 21).

The primer combination *Mse*I-CA + *Eco*RI-AGG showed band polymorphism for the sample ‘44’ from the 15 min IOA ‘Ruby Red’ + 30 Gy ‘Succari’ fusion (Fig. 22).

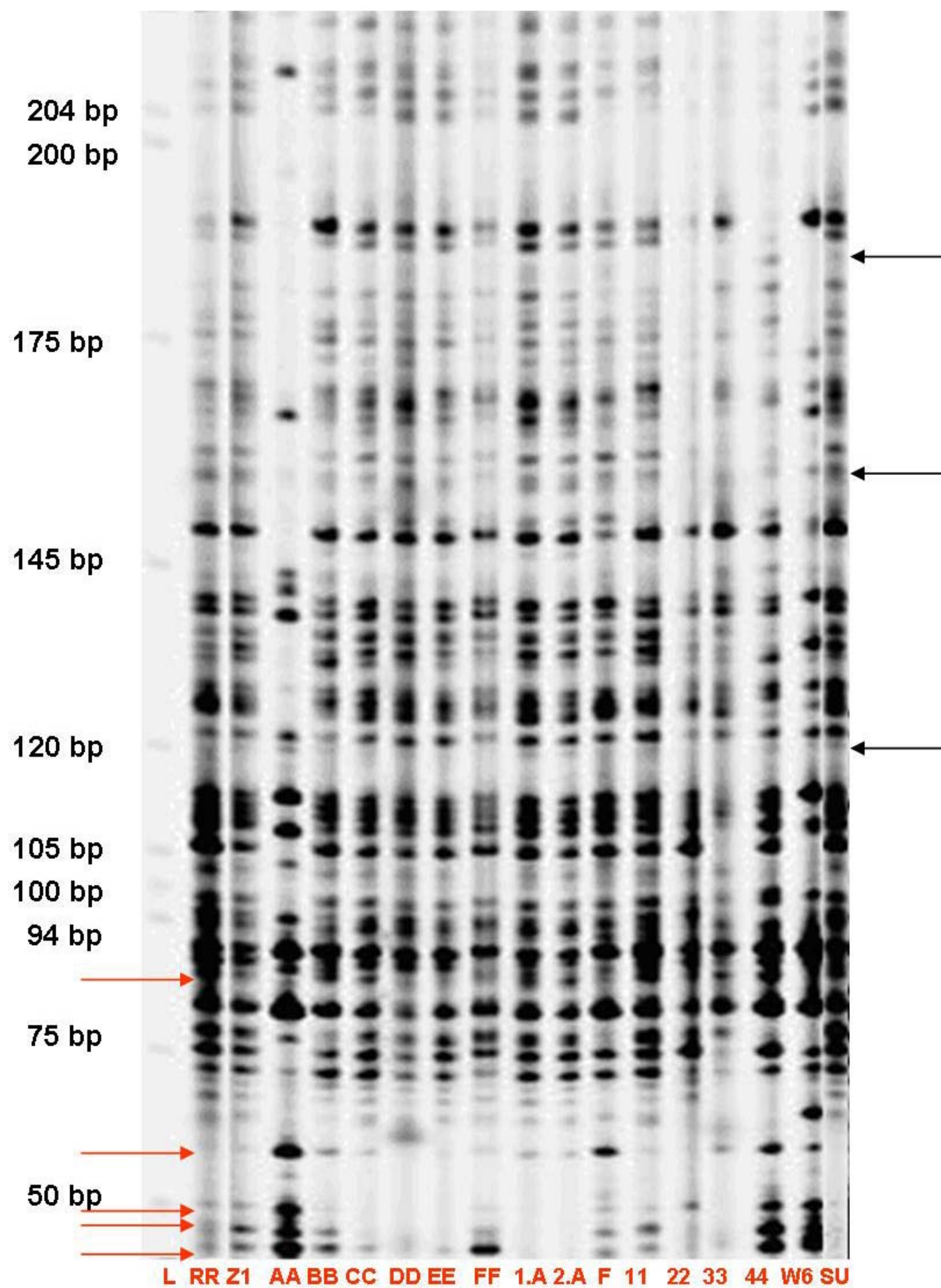

Fig. 19. *Mse*I-CAA plus *Eco*RI-ACA primer combination showing band polymorphism in different 'Ruby Red' + sweet orange fusions. Ladder (L). 'Ruby Red' (RR) and 'Succari' (SU) parents. 20 min 'Ruby Red' + 80 Gy 'Succari' (Z1-EE; 1.1,2.2,W6). 'Itaborai' + 'Ruby Red' (1A,2A). 'Ruby Red' + 100 Gy 'Itaborai' (3A). 20 min 'Rubt Red' + 100 Gy 'Itaborai' (3.3). 15 min 'Ruby Red' + 30 Gy Succari' (4.4).

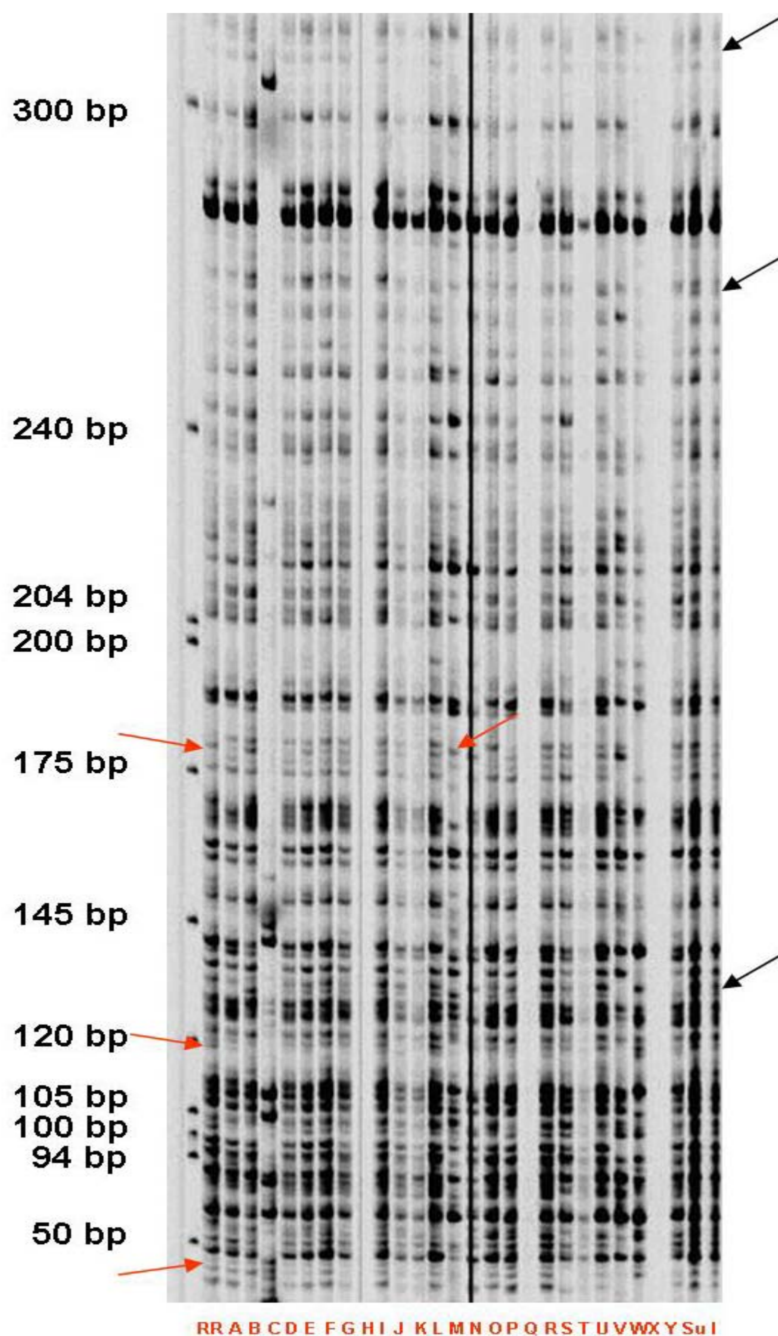

Fig. 20. *Mse*I-CT plus *Eco*RI-ACA primer combination showing band polymorphism in at least nine samples. 'Ruby Red' (RR), 'Succari' (SU) and 'Itaborai' (I) parents. 'Ruby Red' + 100 Gy 'Succari' (B,U). 'Ruby Red' + 100 Gy 'Itaborai' (F,Q). 'Ruby Red' + 'Itaborai' (H,K). 20 min 'Ruby Red' + 80 Gy 'Succari' (A,C,D,E,G,I,J,L,M,N,O,P,R,S,T,V,X,W,Y).

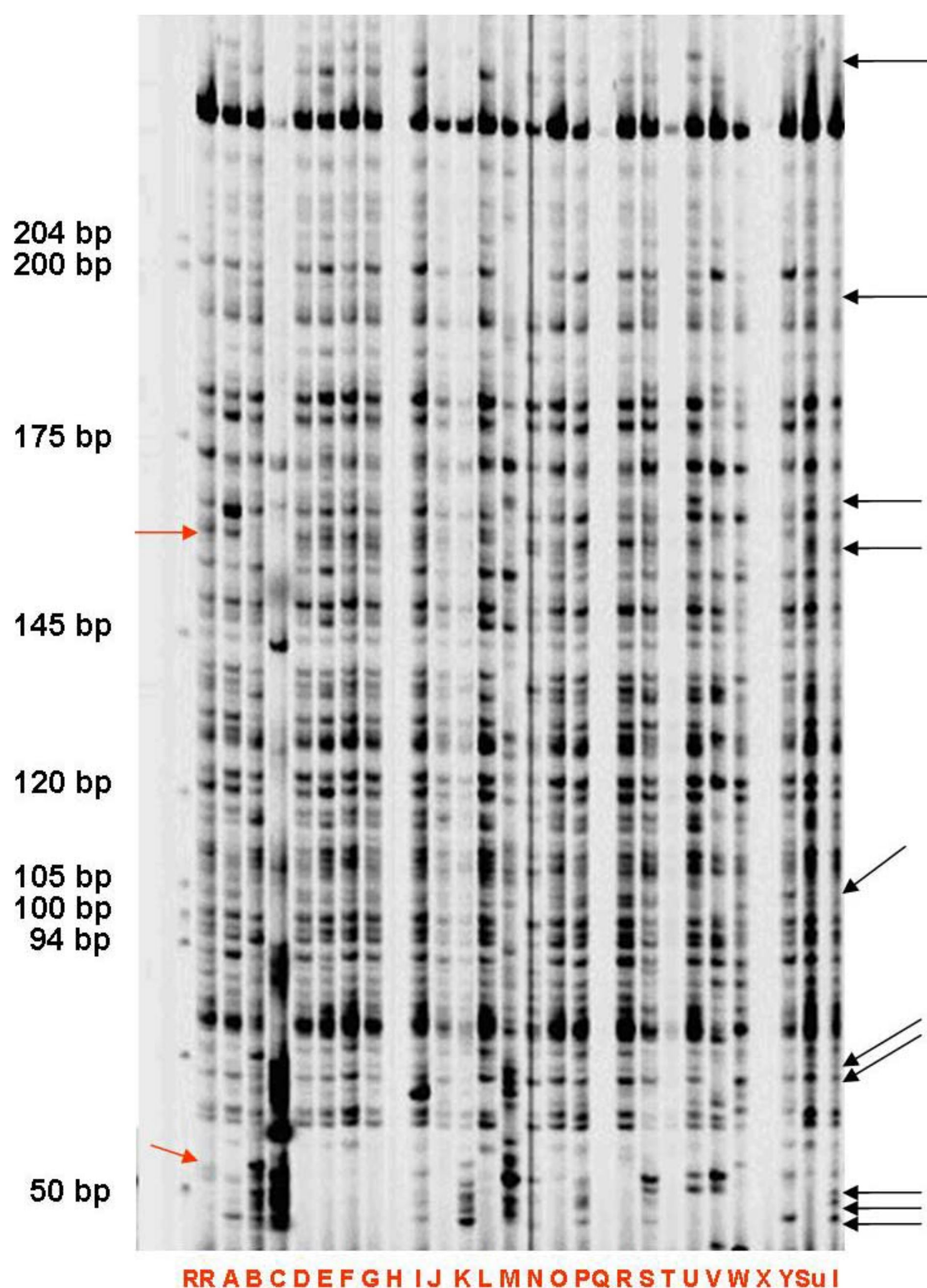

Fig. 21. *Mse*I-CT plus *Eco*RI-ACT primer combinations showing band polymorphism in four samples, three from tetraploid 20 min IOA 'Ruby Red' + 80 Gy 'Succari' plants (E,I,L) and the peculiar ploidy 'Ruby Red' + 100 Gy 'Itaborai' plant (F). 'Ruby Red' (RR), 'Succari' (SU) and 'Itaborai' (I) parents. 'Ruby Red' + 100 Gy 'Succari' (B,U). 'Ruby Red' + 100 Gy 'Itaborai' (F,Q). 'Ruby Red' + 'Itaborai' (H,K). 20 min 'Ruby Red' + 80 Gy 'Succari' (A,C,D,E,G,I,J,L,M,N,O,P,R,S,T,V,X,W,Y).

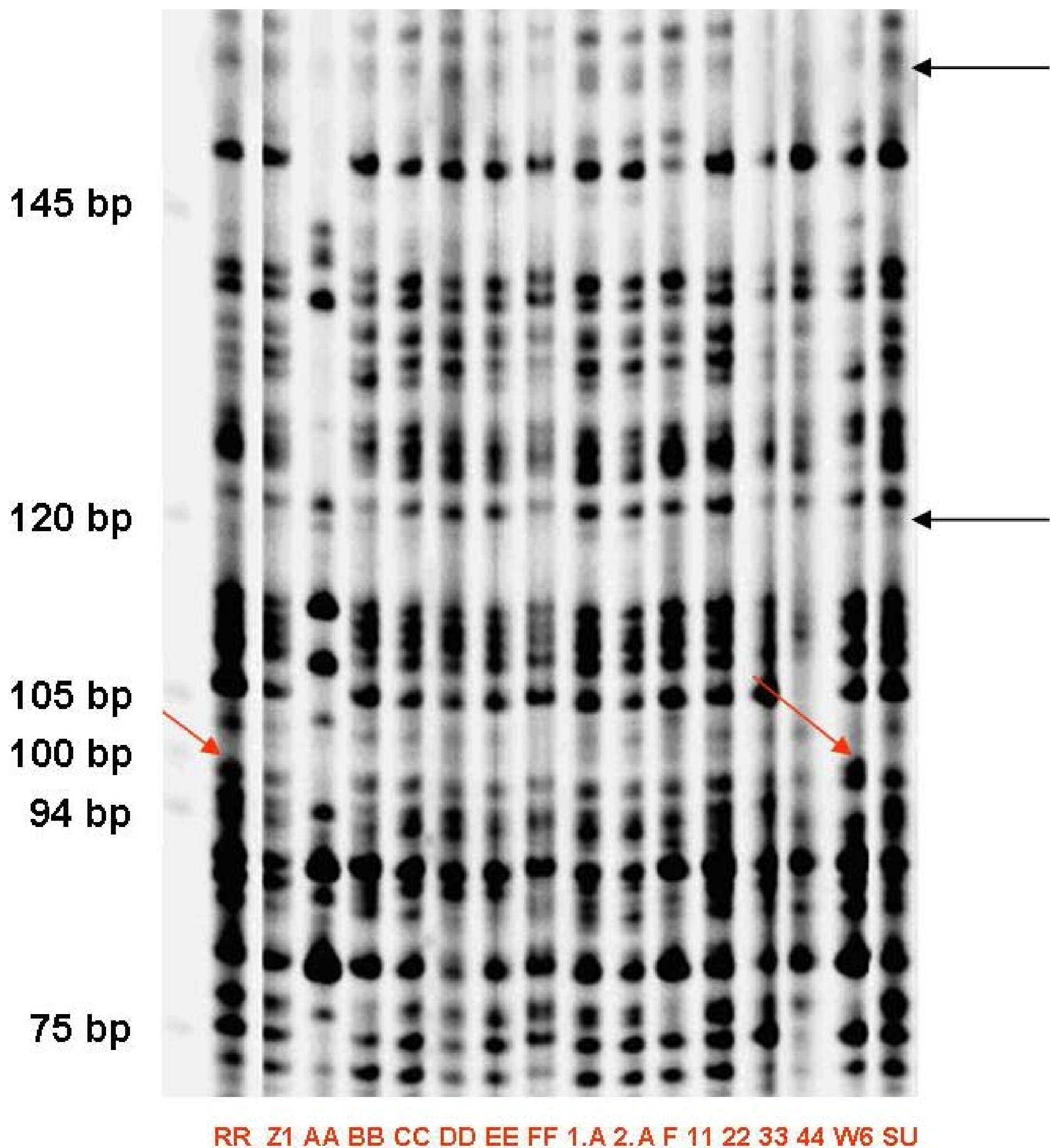

Fig. 22. *Mse*I-CA + *Eco*RI-AGG primer combination showing band polymorphism of sample from the 15 min IOA 'Ruby Red' + 30 Gy 'Succari' fusion. 'Ruby Red' (RR) and 'Succari' (SU) parents. 20 min 'Ruby Red' + 80 Gy 'Succari' (Z1-EE; 1.1,2.2,W6). 'Itaborai' + 'Ruby Red' (1A,2A). 'Ruby Red' + 100 Gy 'Itaborai' (3A). 20 min 'Rubt Red' + 100 Gy 'Itaborai' (3.3). 15 min 'Ruby Red' + 30 Gy Succari' (4.4).

After ploidy confirmation by flow cytometry, samples from the IOA-20 min 'Ruby Red' + 80 Gy 'Succari' tetraploid plants (B,C,D,G,Z9,Z8,K,L,M,O,P), the peculiar 'Ruby Red' + 100 Gy 'Itaborai' (F) and from the 100 Gy 'Itaborai' (I) plant were analyzed for hybridity and genetic difference by comparioson of AFLP based on *Mse*I-CA + *Eco*RI-ACG and *Mse*I-CT + *Eco*RI-ACG primer combinations (Fig. 23 and 24). Band morphology of samples C, G, L and M showed hybridity.

IRRADIATED MICROCELL MEDIATED CHROMOSOME TRANSFER (MMCT)

Proliferating calli were formed from all protoplast-irradiated microptotoplast fusions. However, embryos were only obtained from the 'Ruby Red' plus 70 Gy irradiated *S. glutinosa* microprotoplasts from the first pellet.

DNA was extracted from callus from most of the performed fusions; 'Ruby Red' plus 50 Gy *S. glutinosa* microprotoplasts from the first pellet (H2); 'Ruby Red' plus 70 Gy *S. glutinosa* microprotoplasts from the first, second and third pellet (H3; H4; H5); 'Ruby Red' plus 200 Gy *S. glutinosa* microprotoplasts from the first and second pellet (H6; H7); and 'Murcott' plus 100 Gy *S. glutinosa* microprotoplasts (H8) from the first pellet.

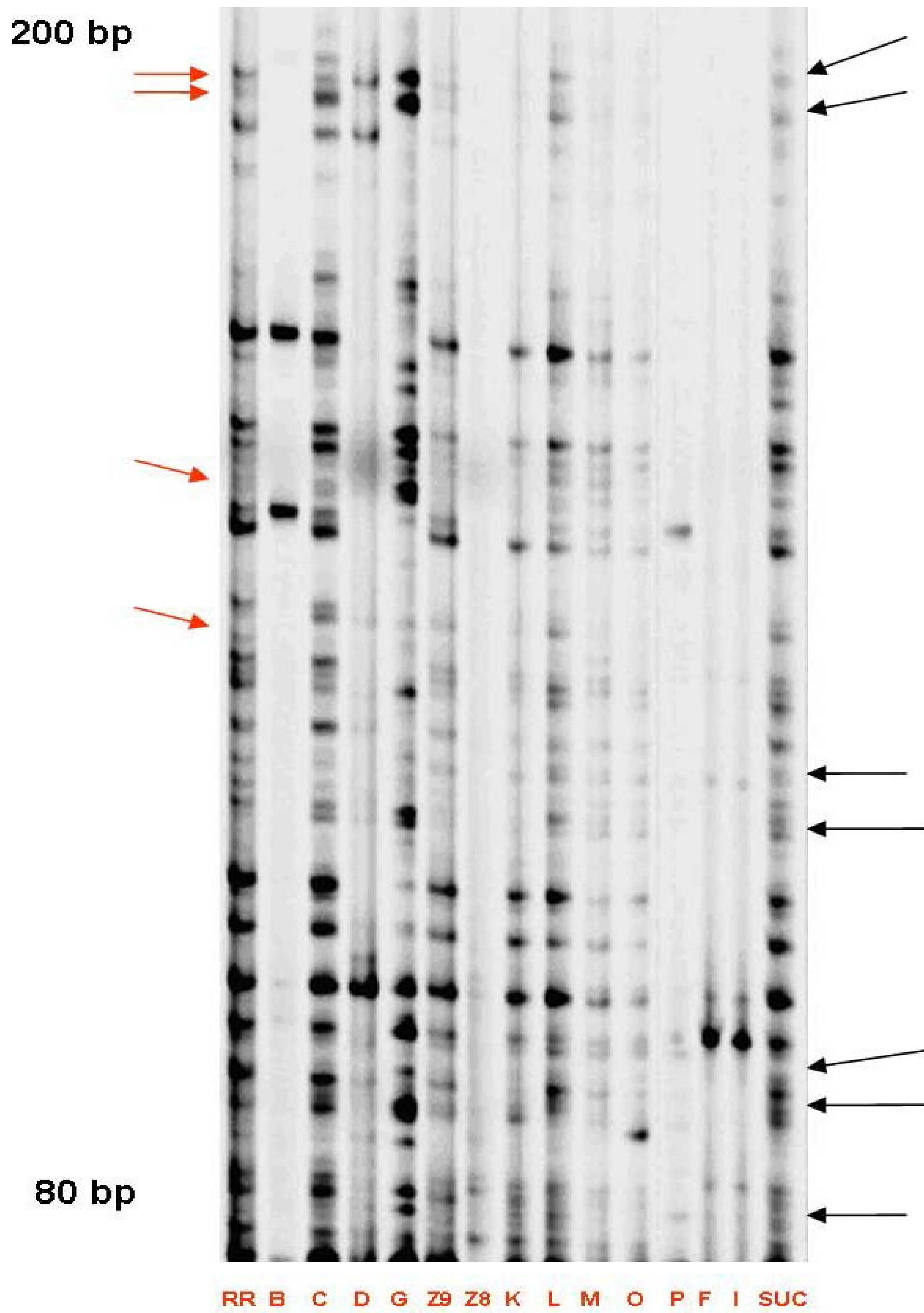

Fig. 23. *Mse*I-CA plus *Eco*RI-ACG primer combination showing band polymorphism in the tetraploid 20 min IOA 'Ruby Red' + 80 Gy 'Succari' fusion plants. 'Ruby Red' (RR) and 'Succari' (SUC) parents.

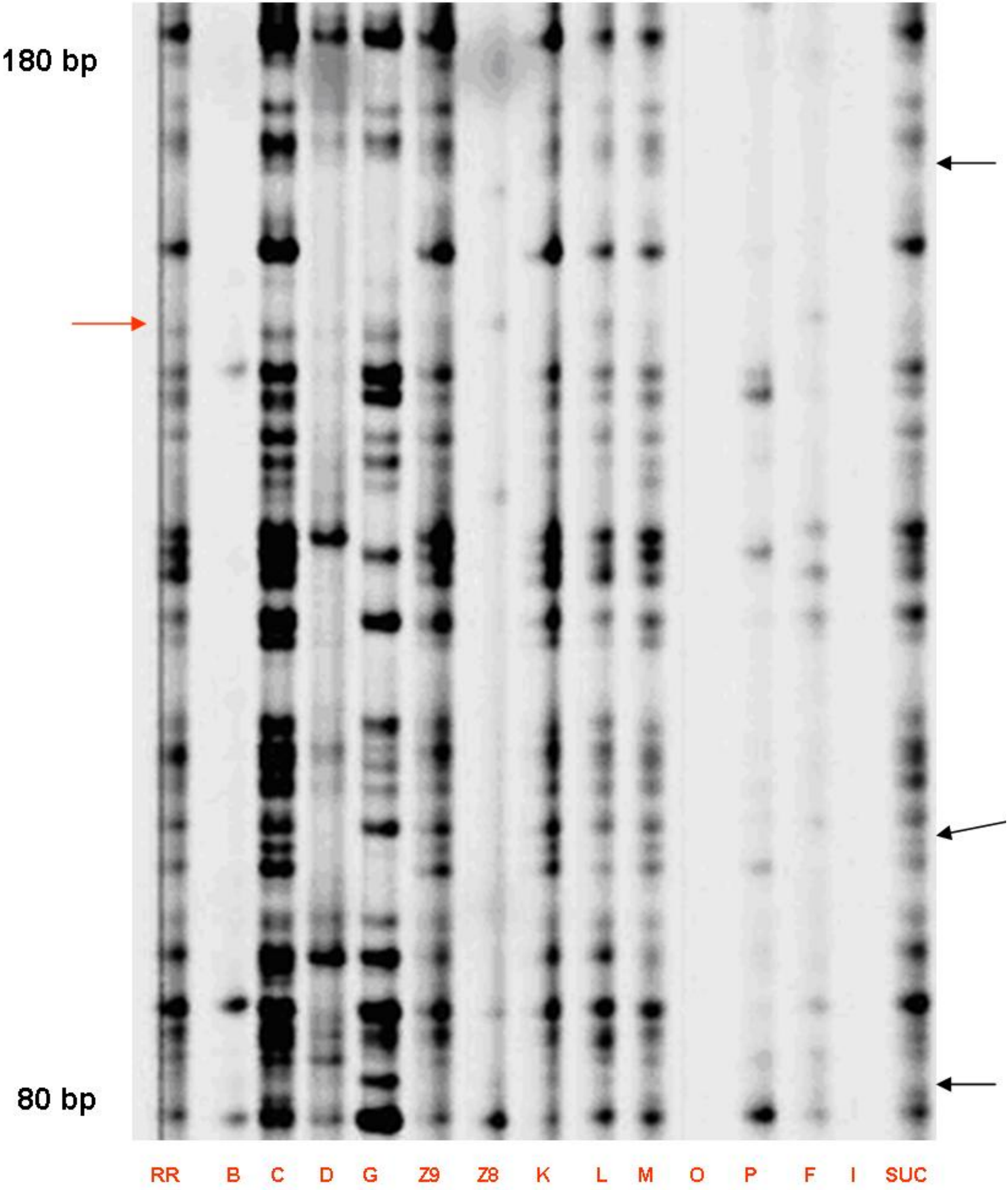

Fig. 24. *Mse*I-CT plus *Eco*RI-ACG primer combination showing band polymorphism in the tetraploid 20 min IOA 'Ruby Red' + 80 Gy 'Succari' fusion plants. 'Ruby Red' (RR) and 'Succari' (SUC) parents.

Dot blot

When 'Murcott' was the target, blocked with excess of 'Murcott' and probed with *S. glutinosa*, all dots showed presence of *S. glutinosa*. Color saturation of the dots were proportional to the amount the probe, target, and blocking DNA used (Fig. 25).

When 'Ruby Red' was the target, blocked with excess of 'Ruby Red' and probed with *S. glutinosa*, all dots showed presence of *S. glutinosa*, and color of the dots was stronger than the above experiment. Color saturation of the dots was proportional to the amount of probe, target, and blocking DNA used (Fig. 26).

When *S. glutinosa* was the target, blocked with excess of 'Ruby Red' or 'Murcott' and probed with *S. glutinosa*, all dots showed, the presence of *S. glutinosa*, and color of the dots was stronger than in both the above experiment. There were no significant differences among the probe, target DNA or proportion of bloking DNA used.

Based on visual observation of the above dot blots, the best target/blocking/probe concentration ratio was chosen. They were 3 µg of target DNA, 5 ng of probe DNA and 1000× blocking DNA.

H8 target DNA, blocked with 'Murcott' and probed with *S. glutinosa*, showed the presence of *S. glutinosa*, as well as both parents (Fig. 27).

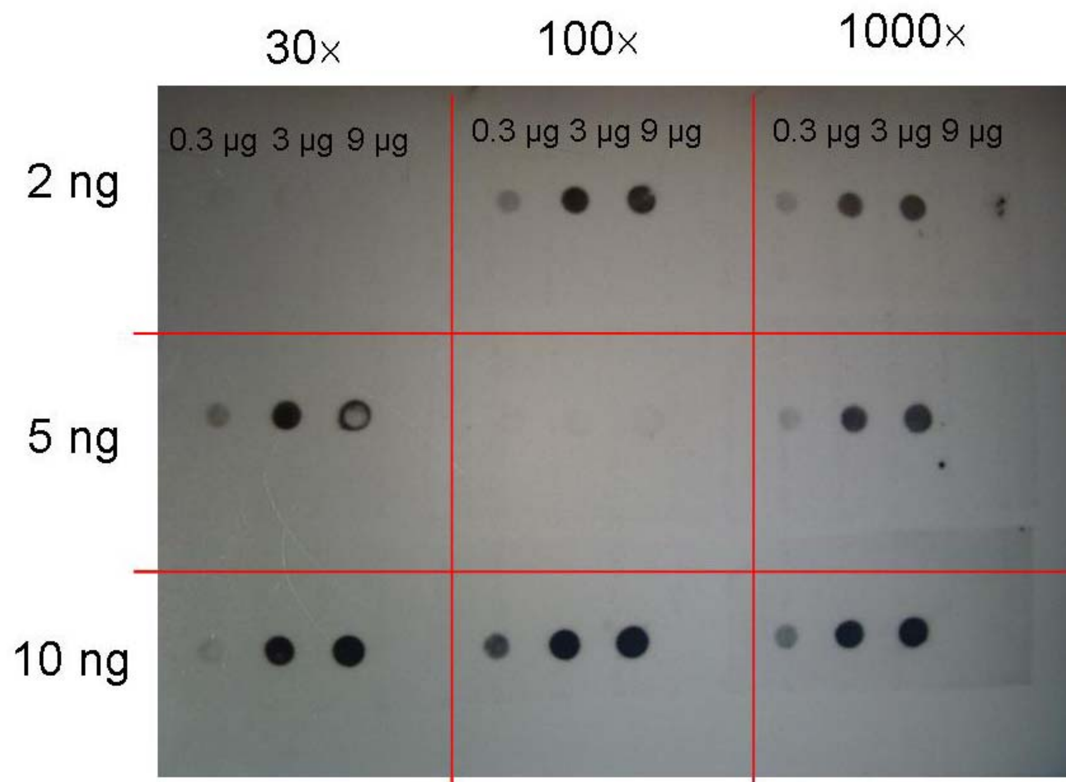

Fig. 25. Dot blot. 0.3, 3 and 9 µg of 'Murcott' DNA blocked with 30, 100 and 1000× 'Murcott' DNA and probed with 2, 5 and 10 ng of *S. glutinosa* DNA.

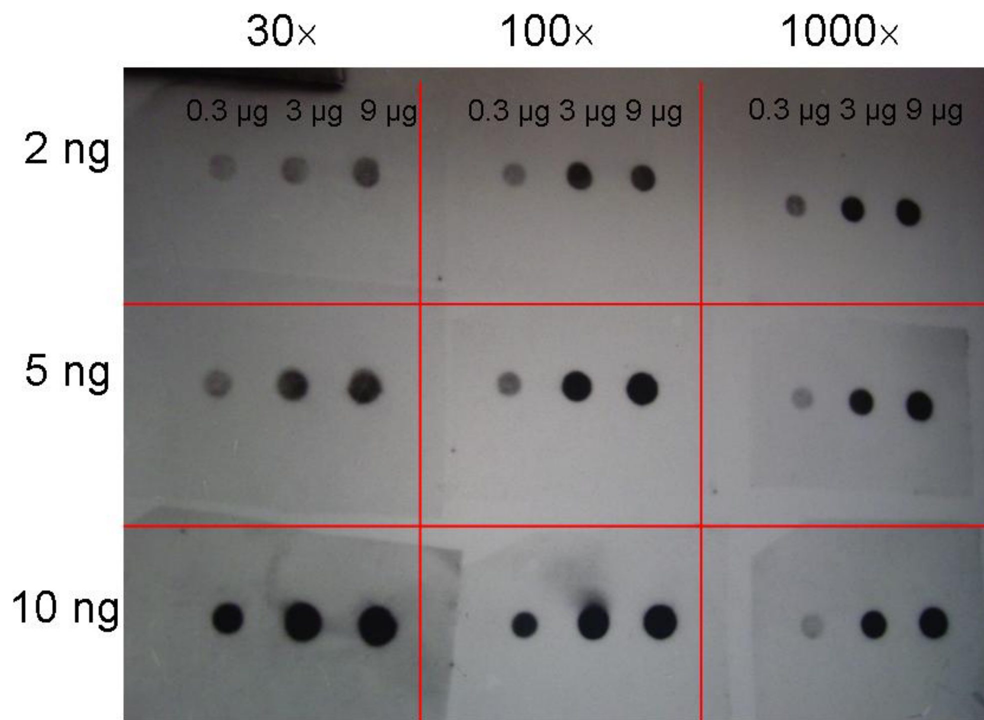

Fig. 26. Dot blot. 0.3, 3 and 9 μg of 'Ruby Red' DNA blocked with 30, 100 and 1000× 'Ruby Red' DNA and probed with 2, 5 and 10 ng of S. glutinosa DNA.

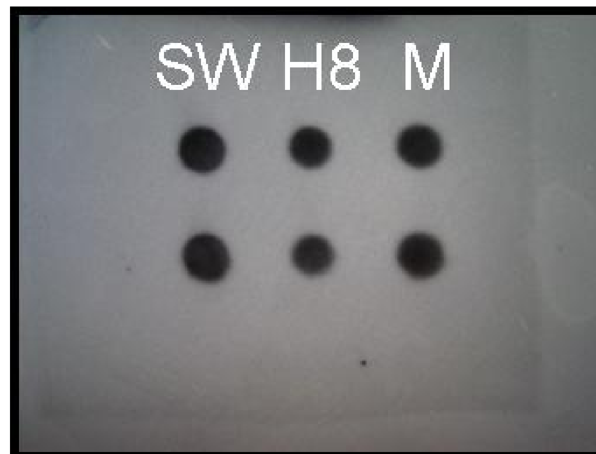

Fig. 27. Dot blot of the hybrid cell line H8, produced by the 'Murcott' (M) protoplast + S. glutinosa (SW) irradiated microprotoplast fusion.

H2, H3, H4, H5, H6 and H7 target DNA, blocked with ''Ruby Red' and probed with *S. glutinosa* showed the presence of *S. glutinosa*. However, color intensity was much lower that both parents (Fig. 28).

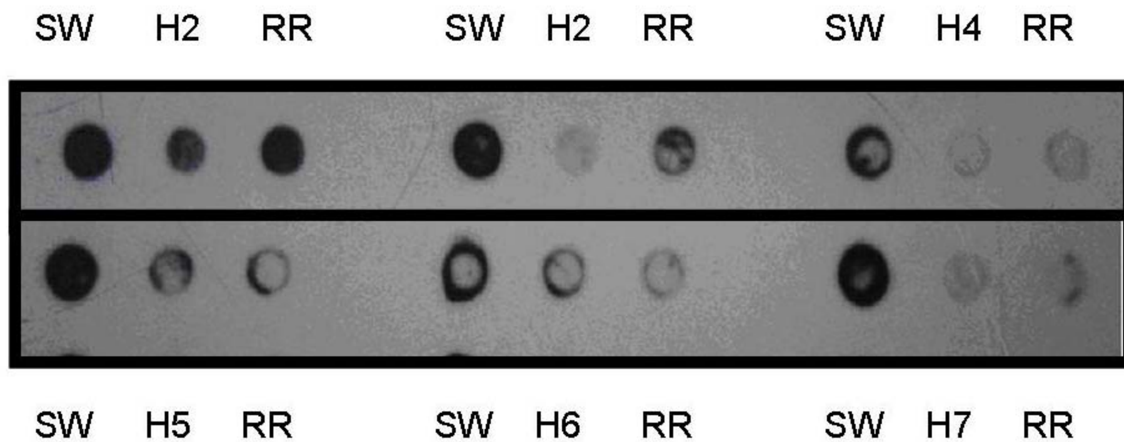

Fig. 28. Dot blot of the hybrid cell lines H2, H3, H4, H5, H6 and H7, produced by the 'Ruby Red' (RR) protoplast + S. glutinosa (SW) irradiated microprotoplast fusion.

AFLP ANALYSIS OF 'MURCOTT' PROTOPLASTS + IRRADIATED S. GLUTINOSA MICROPROTOPLASTS

AFLP analysis showed polymorphic bands.

Bands were complementary from both parents were observed in the H8 cell line (Fig. 29).

The best primer combinations were:

*Mse*I-AC with *Eco*RI-AAC,

*Mse*I-CT with *Eco*RI-ACA,

*Mse*I-CT with *Eco*RI-AAC.

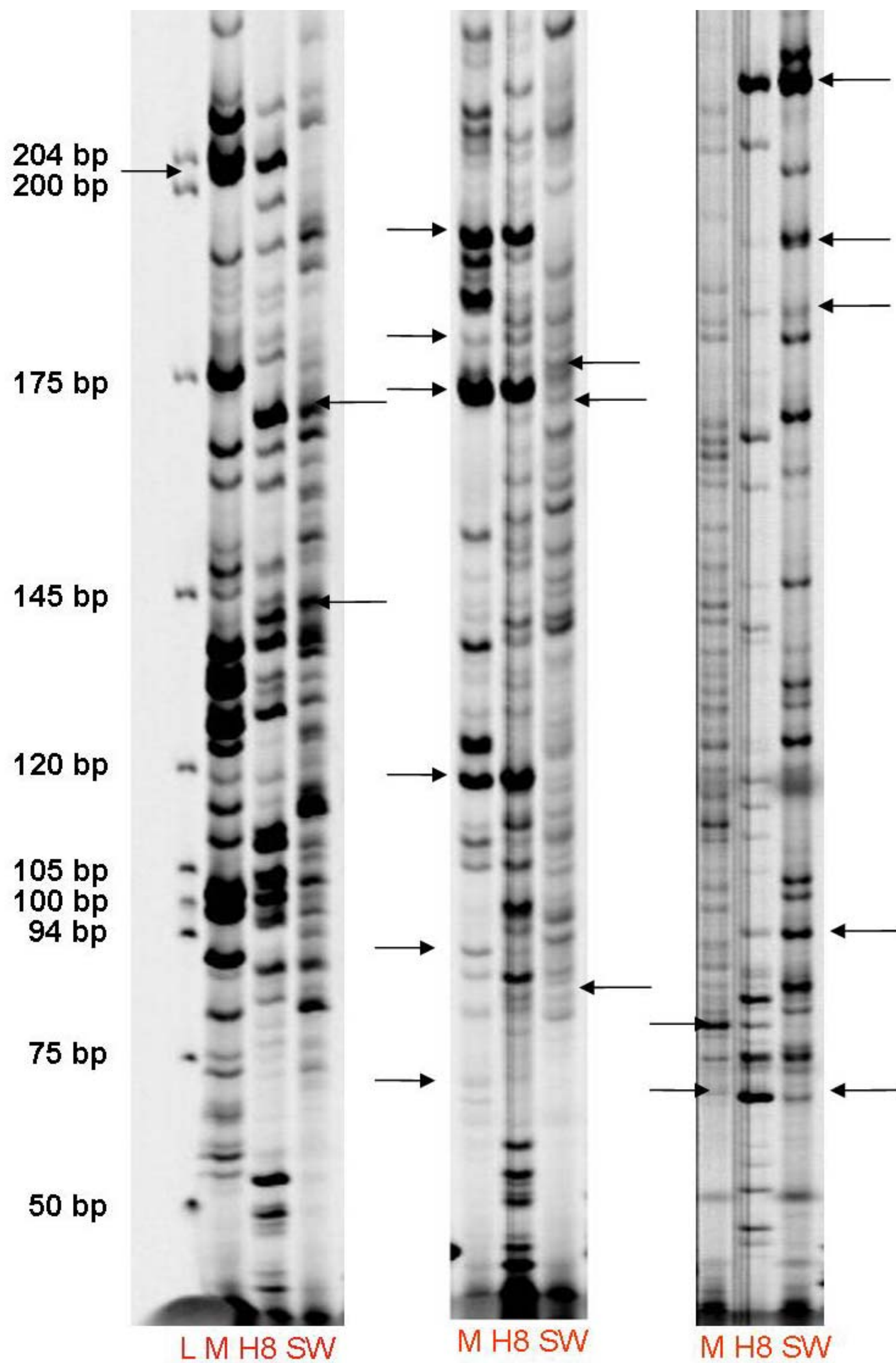

Fig. 29. From left to right. MseI-AC + EcoRI-AAC, MseI-CT + EcoRI-ACA and MseI-CT + EcoRI-AAC primer combinations showing band polymorphism of 'Murcott' (M) protoplast + 100 Gy S. glutinosa (SW) microprotoplast fusion.

AFLP FOR 'RUBY RED' PROTOPLASTS + IRRADIATED S. GLUTINOSA MICROPROTOPLAST

Bands from both parents were shown in the AFLP analysis of H2, H3, H4, H5, H6 and H7 with many primer combinations.

*Mse*I-CAA + *Eco*RI-AGG were the primer combination which showed more *S. glutinosa* insertions (Fig. 30).

The primer combinations *Mse*I-CA + *Eco*RI-ACC and *Mse*I-CT + *Eco*RI-ACT showed two *S. glutinosa* insertions each (Fig. 31 and 32), and the primer combinations *Mse*I-CA + *Eco*RI-ACT and *Mse*I-CT + *Eco*RI-ACT showed one *S. glutinosa* insertions each (Fig. 33).

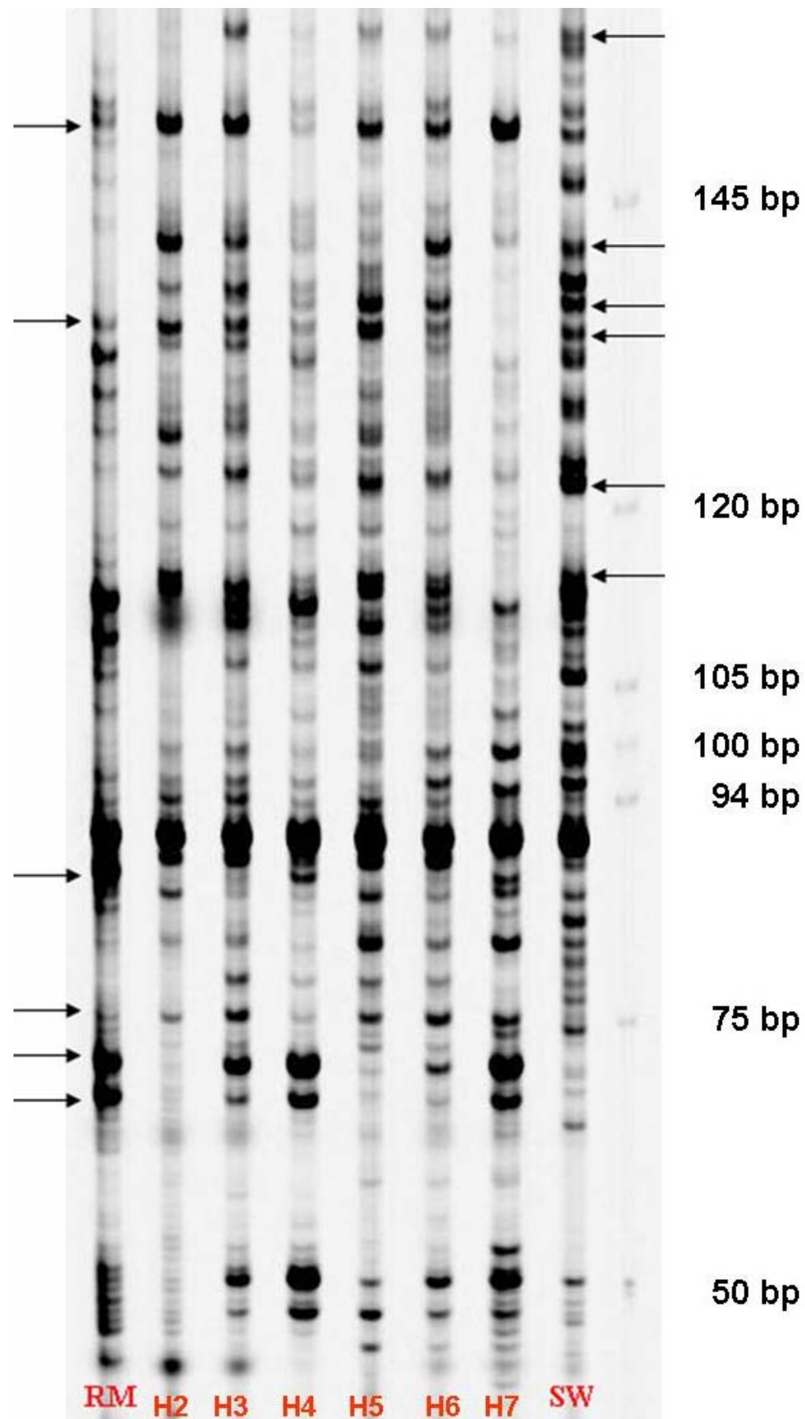

Fig. 30. MseI-CAA + EcoRI-AGG primer combination showing S. glutinosa insertions. ‘Ruby Red’ (RR) and S. glutinosa (SW) parents. Hybrids (H2-H7).

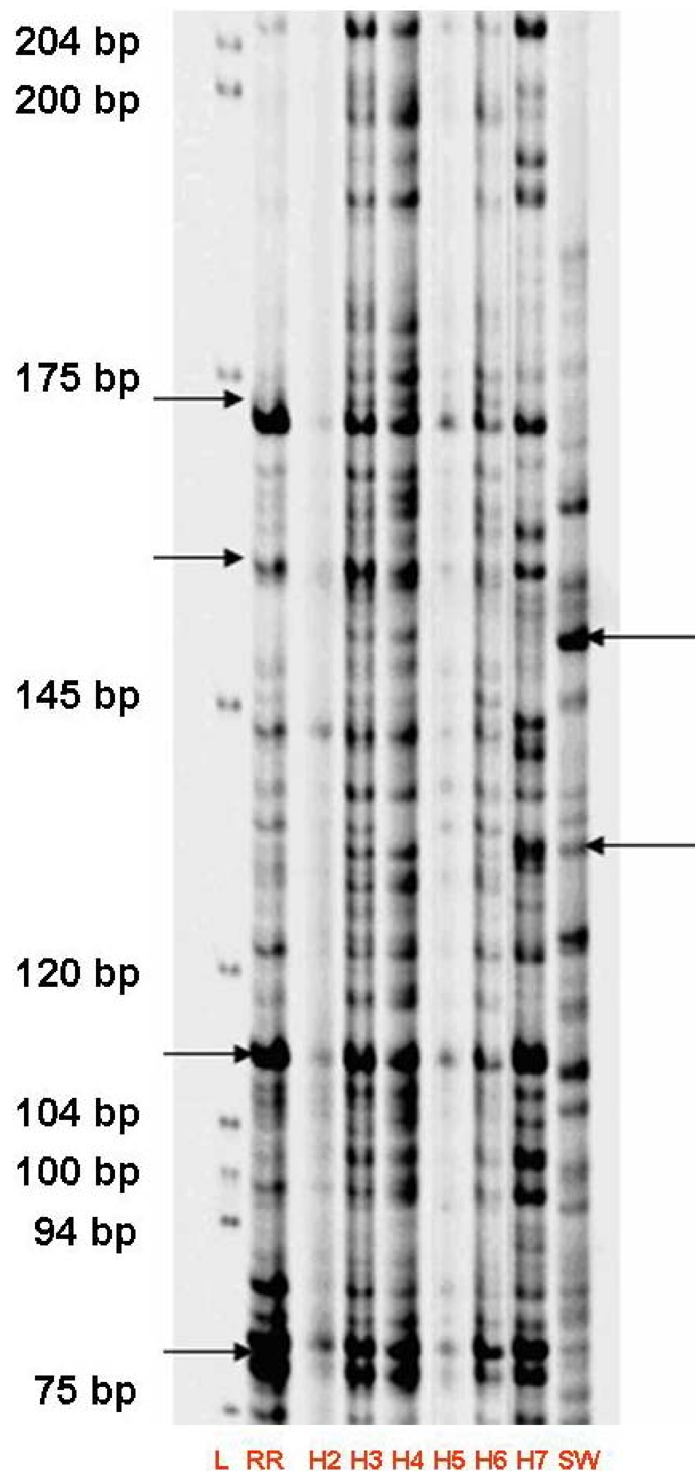

Fig. 31. MseI-CA + EcoRI-ACC primer combination showing two S. glutinosa insertions. ‘Ruby Red’ (RR) and S. glutinosa (SW) parents. Hybrids (H2-H7).

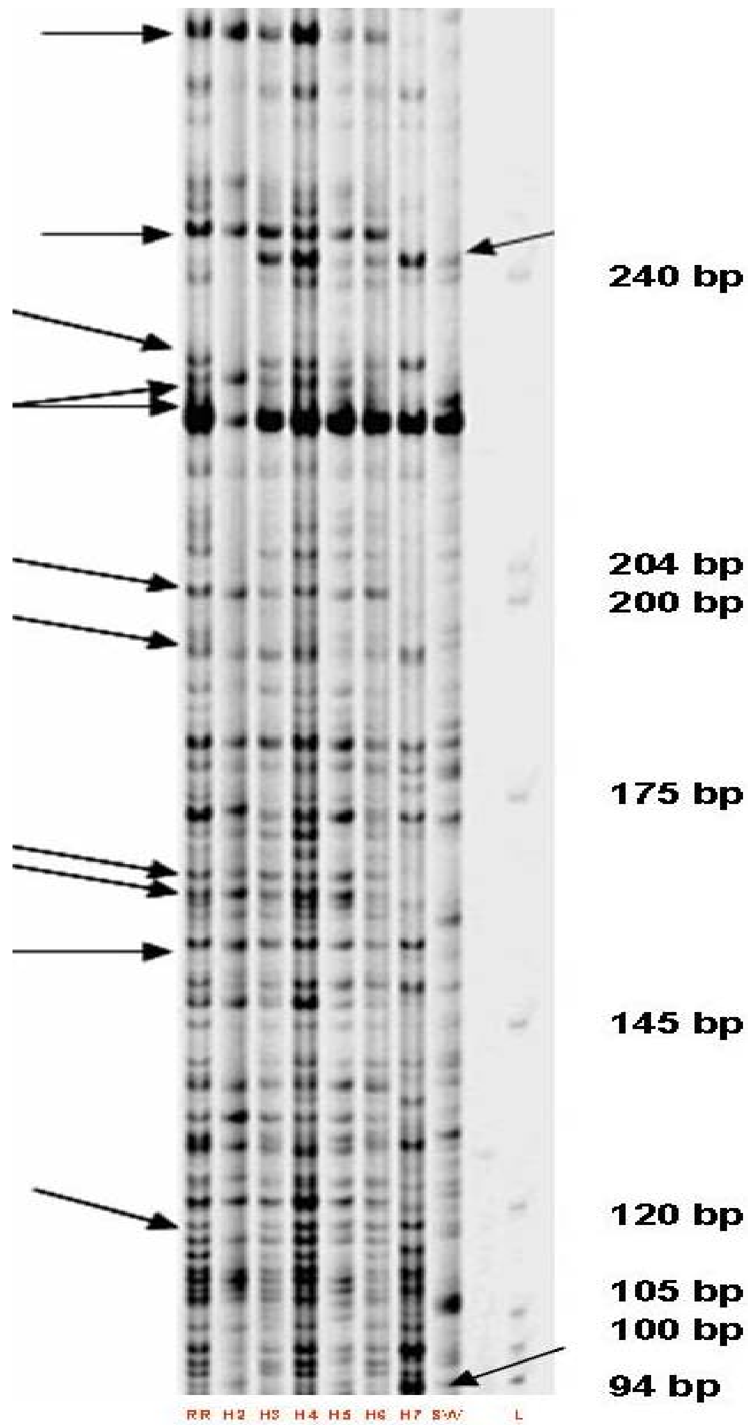

Fig. 32. MseI-CT + EcoRI-ACT primer combination showing two S. glutinosa insertions. 'Ruby Red' (RR) and S. glutinosa (SW) parents. Hybrids (H2-H7).

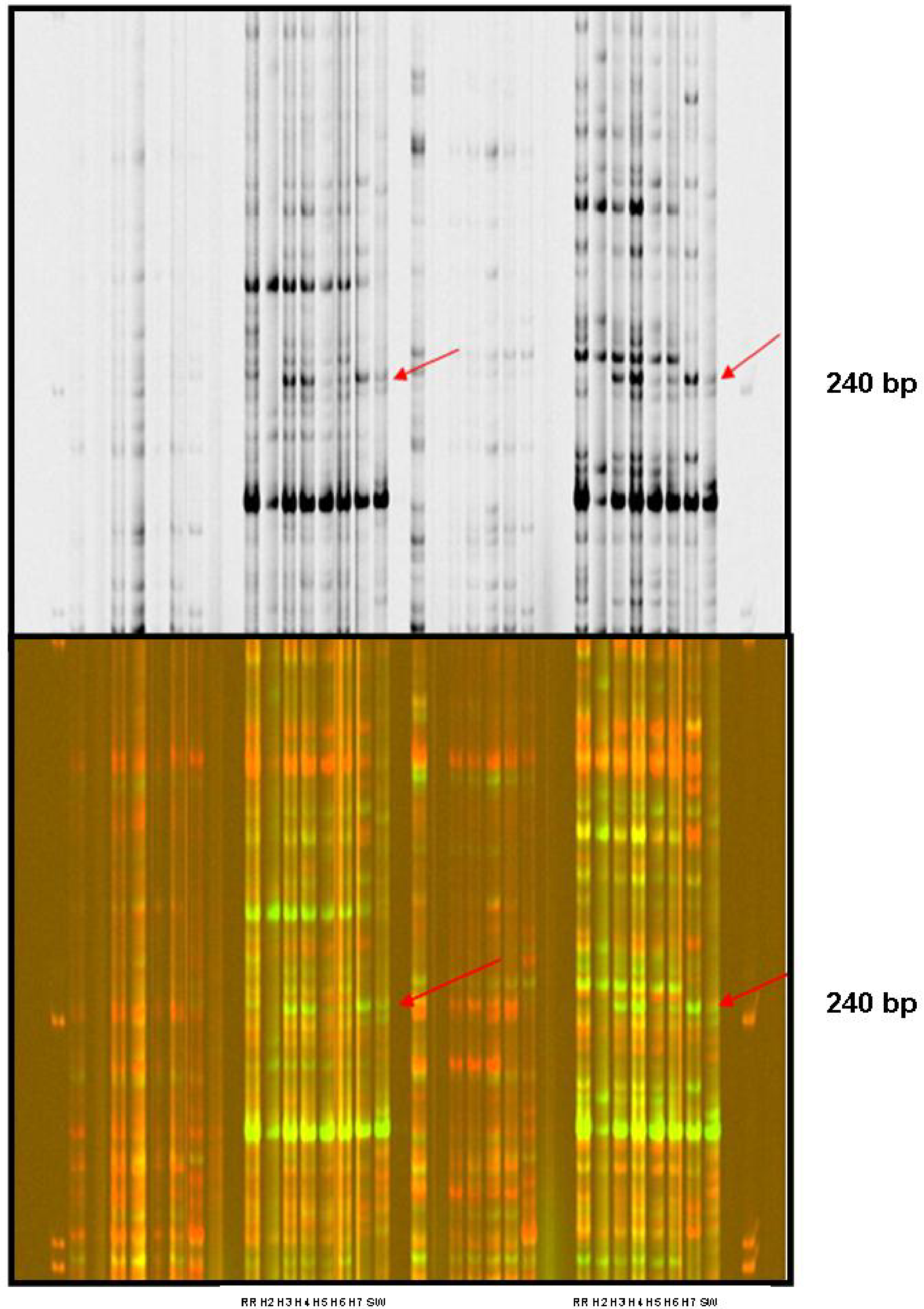

Fig. 33. MseI-CA + EcoRI-ACT and MseI-CT + EcoRI-ACT primer combinations (2nd and 4th AFLP blocks) showing presence of S. glutinosa in the hybrids. 'Ruby Red' (RR) and S. glutinosa (SW) parents. Hybrids (H2-H7).

DISCUSSION

The main goal of this study was to use protoplast fusion to produce symmetric and asymmetric somatic hybrids between grapefruits and sweet oranges and, tangerines and sweet oranges. Such hybrids will create genetic diversity and have potential use in future grapefruit improvement.

The whole process, from protoplast isolation to plant acclimation and somatic hybrid verification was adjusted to increase the chance of success.

The importance of preparing quality protoplast for somatic hybridization by using the right source material could be emphasized (Grosser, 1994). Keeping the suspension cells, as suggested by Grosser and Gmitter (1990), in a 2-week subculture cycle was important to acquire a high yield of quality, low starch protoplasts due to the induction of a logarithmic growth by the frequent media changes. The utilization of suspension cells from one to three days after the last change was important to achieve quantity of protoplasts.

In spite of the efforts to be as consistent as possible in the source material maintenance, there was great difference in protoplast isolation among the different varieties. Some of the species, such as the grapefruits, were easily isolated, while others, such as ‘Hanlim’, were recalcitrant. This was expected, accordingly to Grosser and Gmitter (1999), due to genotypic and/or epigenetic differences among cultivars.

The osmotic pressure of the medium influences both membrane tension and stability. The ideal osmotic pressure for most of the species studied herein was 0.6 M,

which the exception of ‘Murcott’ and ‘Changsha’, in which 0.7 M promoted a better osmotic equilibrium. More than the osmotic equilibrium, the ratio of BH3 medium: enzyme solution had to be individually adjusted to suit each cultivar. Grosser and Gmitter (1999) suggested 0.6 M BH3 when using suspension cells and 0.7 M BH3 when using calli for protoplast isolation. ‘Changsha’ and ‘Murcott’ had too much starch and this may be the reason for needing higher molarity.

A very important modification to the Grosser and Gmitter (1990) protoplast isolation protocol was the gradual digestion by macerozyme (a macerating/separating enzyme) solution for two hours before adding a cellulase plus pectolyase solution, which improved isolation in some of the cultivars. A possible explanation is that cells such pre-digestion allowed a better separation of the cells. Cellulase is a cellulose-digesting enzyme, and pectolyse, a pectin breaker (Fang et al, 2005). The use of macerozymes may have promoted a more efficient infiltration of the cellulose/macerozyme solution.

Before protoplast fusion, some species were exposed to gamma irradiation, to fragment the DNA, or to 3 mM iodoacetamide (IOA) to inactivate metabolic activity.

Protoplast irradiation was very effective in arresting protoplast division, calli and embryo formation. Doses of 150 gamma rays and higher were lethal to the protoplasts. Derks et al (1992) observed some cell division in tomato protoplast using 50 Gy but cell division was completely prevented after 100 Gy. This dose probably exceeded dose leading to fragmentation and subsequent elimination of chromosomes. Protoplasts might have suffered a great harm and a complete division-arrest. Liu and Deng (2002)

observed inhibitory impact on the regeneration of citrus hybrid shoots in a dose-dependent way.

Three mM of IOA, independently of the exposition time, totally inhibited further development of treated cells. Ge et al. (2006), in order to abolish the nursing effect, used 2-4 mM IOA, at least twice the observed 1 mM IOA for 15 min dose found to completely inhibit growth of both wheat and Italian ryegrass protoplasts. However, 1 mM IOA for 20 min at 4 ºC was not sufficient to prevent cell division in chicory protoplasts and 2-4 mM had to be used to completely inhibit protoplast division (Varotto et al., 2001). Furuta et al. (2004) used an even higher dose, 5 mM for 10 min at 4 ºC, to inactivate chrysanthemum protoplasts before fusing with wormwood protoplasts.

Cell division and calli formation were reduced for fusion combinations where one parent was irradiated and also for those where one parent was irradiated and the other treated with IOA, in comparison to fused protoplasts from non-treated fusions. This was probably because treated protoplasts had to recover from damage caused by the treatments. However, once the first stress caused by irradiation and IOA passed, and embryos started to form, there were no significant differences among treated and normal fusions. Formed embryos probably originated from surviving cells produced by genome complementation, since controls alone did not develop. The exception was the 100 Gy 'Itaborai' sweet orange control where two shoots were obtained.

After embryo formation, constant media exchange at least once a month was necessary to regenerate shoots. Fifteen hundred medium and RMAP rooting medium were considered inefficient to respectively induce embryo growth and rooting, therefore,

their usage was discontinued. However, a modified B+ medium with half the normal NAA concentration, placed in magenta boxes, was essential to shoot elongation.

Liu and Deng (2002) produced shoots from irradiated tangerine plus IOA treated tangelo fusion. Nevertheless, shoots were recalcitrant to root, even in root-induction medium.

Some root recalcitrant shoots were grafted onto ‘Rough Lemon’, C-22 or C-146. Later, however, a more proficient solution was determined. The rooting problem was solved by dipping the base of the formed shoots in 3000 $mg.L^{-1}$ IBA for 10 min and placing them in a modified EMEP, with 6% sucrose, in magenta boxes. The longer exposition (10 minutes) to IBA, as well as, the extra sucrose may have helped in rooting formation. Furthermore, because EMEP is a basic medium, conflict of cytokines or auxins were avoided. Furthermore, NAA is a stronger auxin than IBA. Even the smaller concentration may have been toxic to the plants. Liu et al., (2004) compared IBA and NAA effects over *Pueraria lobata* roots and observed that NAA exerted strong inhibition on primary and lateral root elongation.

The size of the shoot when dipped in IBA did not solely determine rooting potential since even tiny shoots formed roots. However, shoots that were bigger were nevertheless more amenable to plant development after planting in soil.

‘Flame’, ‘Valencia’ and ‘Satsuma’ were considered inappropriate for protoplast fusion, since they did not develop further than calli or small embryos.

Shoots were produced from P-P fusions (not exposed to irradiation or IOA), mainly from ‘Ruby Red’ + sweet oranges fusion. Among them (Fig. 11) were two

vigorous diploid shoots which were grafted onto 'Rough Lemon'. Even though there is a possibility that they are cybrids, they are probably fusion escapes because in a normal fusion, donor genomes are not inactivated, and potential escapes may occur.

Cultivars also responded differentially to irradiation. 'Natal' and 'Murcott' appeared to be intolerant of irradiation or IOA treatment and seemed to be a poor choice for future fusions.

Plants from fusions of 'Ruby Red' + sweet oranges were obtained, and 'Itaborai' seemed to be the most resistant to irradiation, since some shoots were formed from the 100 Gy control. A shoot was produced from the 'Ruby Red' + 100 Gy 'Itaborai' fusion and flow cytometry analysis suggests aneuploidy (between triploid and tetraploid). However, the plant is not very vigorous.

The strategy of double inactivation of both parents prior to fusion was very effective. Cell division and calli formation was delayed in irradiation protoplasts compared to non treated ones and avoided with IOA treatment. This was probably because treated protoplasts had to recover from damages caused by irradiation. Liu and Deng (2002) irradiated Dancy tangerine with 228, 342 and 456 Krad and fused with 0.25 mM (15 min) iodoacetic acid treated Page protoplasts. First two doses allowed cell division one month later then the control, and embryo formation (only at 342 Krad) 2 months later. The highest dose took extra 2 months for cell division and 24 months for embryo formation. Formed embryos were probably originated from surviving cells produced by genome complementation, since controls alone did not develop. Accordingly to Grosser and Gmitter (1999) complementation is what allows hybrid

embryos formation following x- or γ- irradiation (donor-recipient fusions). Double inactivation was very robust against escapes in that only one shoot from the 100 Gy 'Itaborai' sweet orange controls was obtained, showing that the produced plants, by genome complementation, were truly hybrids, as later confirmed by AFLP analysis.

Large numbers of embryos and more than 80 plants (mainly from the double inactivation fusions) were produced. Nevertheless, the great majority of the plants (37 in greenhouse and still producing *in vitro*) were produced by the IOA-20 min 'Ruby Red' + 80 Gy 'Succari' fusion. Most of these plants were morphologically similar, with well-expanded leaves, long internodes and a vigorous growth. All plants analyzed by the flow cytometry were tetraploid (Fig. 17).

Double-inactivation was very effective. Time and material were saved. Besides, formed plants were vigorous and the species combination may be very interesting. Hybrid grapefruits expressing higher soluble solids content and lower acidity, (characteristic from 'Succari) would be very valuable for the market, for both fresh and juice production. Furthermore, the tetraploid plants may also be used as breeding parents in interploid crosses.

Such 'Ruby Red' + 'Succari' hybrids are potentially valuable because they would be hardly obtained by sexual hybridization since both species are highly apomictic and polyembryony impairs creation of large segregating population for selection.

Flow cytometry and AFLP analysis were complementary, and their combination was important in identifying the hybrid plants, because one of them showed the ploidy

and the other showed both parents' complementary bands. The AFLP analysis was very conclusive, presenting band polymorphisms in different primer combinations.

PROTOPLAST + IRRADIATED MICROPROTOPLAST FUSION

Similarly to Louzada et al. (2002) work, *S. glutinosa* microprotoplasts were easily isolated, and the procedure was very efficient to produce very small microprotoplasts

Fusions of 'Ruby Red' and 'Murcott' with irradiated *S. glutinosa* may have led to insertions of rearranged chromosomes with the fusion nuclei and/or insertion of *S. glutinosa* DNA into chromosomes of 'Ruby Red'and 'Murcott'. Representation of *S. glutinosa* genome in a given product would presumably be reduced due to the use of microprotoplasts. This may be important as a first step for a future radiation mapping of Citrus.

Dot blot was not considered a very explanatory analysis in this work because, even tough presence of *S. glutinosa* could be detected on the dots, it was not visually ideal.

CONCLUSIONS AND SUGGESTIONS

Procedures were developed to improve protoplast fusion protocol by combining it with double genome inactivation (irradiation and IOA), and altering cultivation media. Rooting recalcitrance problem was solved and all produced plants using double inactivation are potentially hybrids saving time and money in the selection process. The produced hybrid plants may have great potential as both superior hybrids presenting desirable characteristics from both parents and as tetraploid parents in interploid crossings.

Furthermore, for the first time *S. glutinosa* microprotoplasts were irradiated and fused to 'Ruby Red' or 'Murcott' protoplasts and all produced calli are hybrids AFLP analysis confirmed presence of *S. glutinosa* into the receptor genomes, showing a possible donor introgression. This technique may be a first step for future for radiation mapping in *Citrus*.

Because citrus chromosomes are small, 2µ in size (Usman, 2006), and morphologically undistinguishable it is suggested that active growing apices be used for chromosome counting as a way to assess chromosome number and detect possible chromosome loss. Furthermore, genomic *in situ* hybridization (GISH) is suggested. GISH has several key steps affecting the final result and requires high-quality chromosome slides (Fu et al., 2004). However, after selecting the hybrid plants which present potential value, it would be interesting to invest time to perform GISH analysis, because the technique may allow not only for an effective chromosome number

determination (possible chromosome pieces, translocations and duplications may potentially interfere in chromosome countings) but also for visualization of chromosomes from each donor species, as previously observed by Fu et al. (2004) in intergeneric somatic hybrids of Goutou sour orange and *Poncirus trifoliate*.

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APPENDIX A

H + H medium

Ingredient	For 1 liter
Macronutrient stock*	10 mL
BH3 macronutrient stock**	5 mL
Vitamin stock***	10 mL
Calcium stock****	10 mL
Iron stock*****	15 mL
Sucrose	5 mL
Malt extract	50 g
Glutamine	1.55 g
pH to 5.8 with Potassium hydroxide (KOH)	

*82.5 g NH_4NO_3; 95 g KNO_3; 18.5 g $MgSO_4.7H_2O$; 7.5 g KH_2PO_4; 1 g K_2HPO_4.
**37 g $MgSO_4$; 15 g KH_2PO_4; 2 g H_2HPO_4; 150 g KCl.
***10 g mio-inositol; 1 g thiamine-HCl; 1 g pyridoxine-HCl; 0.5 g nicotinic acid; 0.2 g glycine.
****29.33 g $CaCl_2.2H_2O$.
*****7.45 g Na_2EDTA; 5.57 g $FeSO_4.7H_20$.

APPENDIX B

BH3 medium

Ingredient	For 1 liter
BH3 macronutrient stock	10 mL
Micronutrient stock*	10 mL
Vitamin stock	10 mL
Calcium stock	10 mL
Iron stock	15 mL
Vitamin A stock**	2 mL
Vitamin B stock***	1 mL
KI stock****	1mL
Sugar alcohol stock*****	10 mL
Organic acid stock******	20 mL
Coconut water	20 mL
Sucrose	51.345 g
Malt extract	1 g
Glutamine	3.1 g
Mannitol	81.99 g
Casein enzyme hydrolysate	0.25 g
pH to 5.7 with Potassium hydroxide (KOH)	

*0.62 g H_3BO_3; 1.68 g $MnSO_4 . H_2O$; 0.86 g $ZnSO_4 . 7H_2O$; 0.083 g KI; 0.025 g $Na_2MoO_4 . 2H_2O$; 0.0025 g $CuSO_4 . 5H_2O$; 0.0025 g $CoCl_2 . 6H_2O$.
**0.05 g calcium pantothenate; 0.1 g ascorbic acid; 0.05 g choline chloride; 0.001 g p-aminobenzoic acid; 0.02 g folic acid; 0.01 g riboflavin; 0.001 g biotin.
***0.001 g retinol; 0.001 g cholecalciferol; 0.02 g vitamin B12.
****0.075 g KI.
*****2.5 g fructose; 2.5 g ribose; 2.5 g xylose; 2.5 g mannose; 2.5 g rhamnose; 2.5 g cellobiose; 2.5 g galactose; 2.5 g mannitol.
******0.1 g sodium pyruvato; 0.2 g citric acid; 0.2 g malic acid; 0.2 g fumaric acid.

APPENDIX C

PEG solution

Ingredient	For 100 mL
PEG	40 g
$CaCl_2$	0.97 g
Glucose	5.41 g
pH to 6 with Potassium hydroxide (KOH)	

APPENDIX D

Solution A

Ingredient	For 100 mL
DMSO	10 mL
CaCl2	0.97 g
Glucose	7.2 g
pH to 6 with Potassium hydroxide (KOH)	

APPENDIX E

Solution B

Ingredient	For 100 mL
Glycine pH to 10.5 with Potassium hydroxide (KOH)	2.25 g

APPENDIX F

Solution 1:1:1

Ingredient
1 part 0.6 M BH_3
1 part 0.5 M EMEP
1 part 0.146 M EMEP

APPENDIX G

Solution 1:2

Ingredient
1 part 0.6 M BH3
2 parts 0.146 M EMEP

APPENDIX H

EMEP solid medium

Ingredient	For 1 liter
Macronutrient stock	20 mL
Micronutrient stock	10 mL
Vitamin stock	10 mL
Calcium stock	15 mL
Iron stock	5 mL
Sucrose	50 g
Malt extract	0.5 g
Agar	8 g
pH to 5.8 with Potassium hydroxide (KOH)	

APPENDIX I

1500 medium

Ingredient	For 1 liter
Macronutrient stock	20 mL
Micronutrient stock	10 mL
Vitamin stock	10 mL
Calcium stock	15 mL
Iron stock	5 mL
Sucrose	50 g
Malt extract	1.5 g
Agar	8 g
pH to 5.8 with Potassium hydroxide (KOH)	

APPENDIX J

B+ medium

Ingredient	For 1 liter
Macronutrient stock	20 mL
Micronutrient stock	10 mL
Vitamin stock	10 mL
Calcium stock	15 mL
Iron stock	5 mL
Sucrose	25 g
Coumarin stock*	10 mL
NAA stock**	72 µL
Agar	8 g
pH to 5.8 with Potassium hydroxide (KOH)	
Gibberelic acid (GA_3) after autoclaving	1 mL

*1.46 $g.L^{-1}$.
**279.3 $mg.L^{-1}$

APPENDIX K

DBA3 medium

Ingredient	For 1 liter
Macronutrient stock	18 mL
Micronutrient stock	9 mL
Vitamin stock	9 mL
Calcium stock	15 mL
Iron stock	5 mL
Coconut water	20 mL
Sucrose	12.5 g
2,4-D stock*	10 µL
6-BAP stock**	3 mL
Agar	8 g
pH to 5.8 with Potassium hydroxide (KOH)	

*66.3 $mg.L^{-1}$
**3 $\mu g.L^{-1}$.

APPENDIX L

RMAP (rooting) medium

Ingredient	For 1 liter
Macronutrient stock	10 mL
Micronutrient stock	5 mL
Vitamin stock	5 ml
Calcium stock	15 ml
Iron stock	5 ml
NAA stock*	72 μLl
Sucrose	12.5 g
Activated charcoal	0.5 g
Agar	8 g
pH to 5.8 with Potassium hydroxide (KOH)	

*20 $\mu g.L^{-1}$

VITA

Claudine Bona received a B.S degree in Agronomy in 1998 and a M.S. degree in Agronomy – Plant production in 2002, both from Parana Federal University, Brazil. She received her PhD in Horticulture from Texas A&M University, United States, in May, 2007.

Her area of expertise includes agronomy, with emphasis on medicinal and aromatic plants and fruit production.

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- Tissue culture of fruit from a temperate climate, especially grapes, and of fruits tropical climate, especially citrus. Specialized in symmetric and asymmetric somatic hybridization, microcell mediated chromosome transfer (MMCT), and some experience in molecular biology.

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