Effectiveness of Gamma Rays on Virus and Viroid Diseases of Monreal Clementine and its Recovery by Shoot-Tip Grafting

A. Caruso, M. Davino, G. Terranova, A. Starrantino, R. Marino, and B. Torrisi

ABSTRACT. To obtain trees of Monreal Clementine mandarin which produce seedless fruits, budwood of this cultivar was exposed to different amounts of gamma rays (0, 2, 4, 6, 8 and 10 kR). From the 223 plants obtained, three clones were selected showing valuable agronomic characteristics besides being almost seedless. Concomitantly, the viral and viroid presence was investigated. The mother trees were found to be infected with psorosis-A (CPs-AV), citrus ringspot virus (CtRSV), citrus exocortis viroid (CEVd), citrus cachexia viroid (CCaVd) and citrus viroid IIId (CVdIIId). Trees obtained from budwood exposed to gamma rays maintained the same viral presence but, at high radiation dosages, the viroid profile was changed with the disappearance of CCaVd and CVIIId. Three selections considered valuable underwent shoot-tip grafting (STG) and the resultant plants maintained their agronomic behavior. Thus far, these selections have produced well in areas where the Comune Clementine is unsuitable. The fruits have few seeds (2-3 per fruit), a dark red skin and do not senesce as those of Comune Clementine do, thus permitting a delay of the harvesting period until to end of January. These clones were released a few years ago and are included in the Italian Voluntary Certification Program.

Index words. Italian Voluntary Certification Program, Biological indexing, dPAGE.

Italian citriculture includes many varieties of both native and recently introduced cultivars of orange, lemon, mandarin, mandarin-hybrids and grapefruit. Various surveys have shown that old citrus plantings in Italy is infected with various viruses, viroids and virus-like diseases (3, 13). The different commercial needs, as well as the danger of future infection by severe citrus tristeza virus (CTV), have encouraged researchers to produce certified citrus cultivars with desirable characteristics and be free of viruses, viroids and other graft-transmissible pathogens in order to propagate citrus on rootstocks other than sour orange.

The cultivation of Clementine mandarin in Italy has increased very rapidly in recent years, totaling 20,000 ha with a production of 320,000 tons in 1994. Clementines are produced throughout southern Italy with the main belt located in Calabria in the Rossano and the Corigliano-Sybaris plain. The cultivars usually grown are Comune, Monreal and, in lesser quantities, di Nules and Oroval.

The Comune is the cultivar most commonly grown in Italy because it lacks seed and generally has good yields. However, it does not produce well in some citrus areas and has the problem of senescence if it is not picked in time. Our objective was, therefore, to improve the agronomic and phytosanitary qualities of Monreal Clementine, since initial observations indicate that this clone has good adaptability to the different Italian citrus areas. Among its negative characteristics are excessive seediness and heavy virus and viroid infections, which limit its propagation on rootstocks different from sour orange.

Considering that gamma rays have been effective in the inactivation of CTV (6), and at the same time induced mutagenesis on the irradiated material (10, 11), we tested gamma ray therapy to evaluate its effect on viruses and viroids and to attempt to obtain virus and viroid-free trees of Monreal Clementine

with good fruit quality and low seed count.

MATERIALS AND METHODS

Monreal Clementine trees grafted on sour orange rootstock were used for our trials. Since 1960, these trees were grown in the experimental field of Palazzelli (Lentini - SR) of Istituto Sperimentale per l'Agrumicoltura di Acireale (ISA). The mother trees showed clear ringspot symptoms on leaves and fruits.

For biological indexing, a minimum of 10 budsticks were collected from each candidate mother tree, from trees obtained after exposure to gamma rays and from plants after shoot-tip grafting (STG). Pineapple and Madam Vinous sweet orange seedlings were graft-inoculated for detection of citrus psorosis-A virus (CPs-AV) and citrus ringspot virus (CtRSV). Nucellar Femminello Fior d'Arancio lemon grafted on sour orange was used for detection of citrus variegation virus (CVV) and citrus crinkly leaf virus (CCLV). Etrog Arizona 861-S-1 citron grafted on sour orange was used for the detection of the citrus exocortis viroid (CEVd) and for other citrus viroids (CVds). Parson's special mandarin grafted on sour orange rootstock was the indicator for the citrus cachexia viroid (CCaVd). Two inoculum pieces were grafted to each of the indicator plants. For each assay, eight inoculated and two non-inoculated controls of 2-yr-old plantlets or seedlings were used and were grown at 20 to 24°C for virus detection and 26 to 32°C for viroid detection (9).

Extractions and electrophoretic analysis to ascertain the presence of viroids were performed on all the samples according to the methods of Albanese et al., (1) and La Rosa et al. (7). Denaturing polyacrylamide gel electrophoresis (dPAGE) analysis was performed with CEVd RNA used as a marker. Gels were stained with silver nitrate. All plants to be used as mother plants were re-tested according to the method suggested by Flores and Llacer (5) which uses filtration through CF-11 cellulose.

For gamma ray treatment, 1-yr-old budsticks, containing four buds each were collected from the Monreal Clementine mother tree and immediately transferred to the Comitato Nazionale per la Ricerca e per lo Sviluppo dell'Energia Nucleare e delle Energie Alternative (ENEA) and irradiated with gamma rays emitted by ^{60}Co. Forty budsticks were treated with each of the following dosages: 0, 2, 4, 6, 8 and 10 kR (10). Three lines were selected out of 223 treated plants (11). Selection was based on good agronomic qualities and low seed counts (2-3 per fruit).

The three lines obtained after the treatment with gamma rays which showed the combined good qualities of productivity, size, low number of seeds, juice yield, persistence of fruit on the tree, etc. were re-tested for viruses and viroids as previously described. Because of heavy virus and viroid content, they were subjected to STG by the methods of Navarro et al. (8) and Starrantino (12). Integuments were removed from the seeds of Troyer citrange which was used as rootstock and were sown in solid Murashige-Skoog (MS) medium and grown at 27°C in the dark. After 15 days, the young seedlings were micrografted under aseptic conditions. The shoot tips consisted of the apical meristem plus the first primordial leaves. The micrografted plantlets were transferred to liquid MS medium and incubated at 27°C under 1,000 lux light for 16 h per day. When the micrografted apex reached the height of 3-5 cm, the small plantlets in the tubes were grafted on 2 to 3-yr-old sour orange seedlings to force the grafted shoot tip by the method of de Lange (2). The growing STG plants thus obtained were re-indexed for virus and viroid infections.

RESULTS AND CONCLUSIONS

The biological and electrophoretic assays confirmed that Monreal Clementine mother trees were infected with CPs-AV, CtRSV, CEVd, CCaVd and CVdIIId. Pineapple and Madam Vinous sweet orange seedlings showed vein flecking and yellowish rings with green islands on some leaves 3-4 mo after inoculation. Femminello Fior d'Arancio lemon indicators showed no symptoms of CVV or CCLV. Citrons showed severe symptoms of epinasty on leaves of young shoots and Parson's special mandarin showed wood pitting and gum impregnations at the first internode 8-12 mo after inoculation. The d-PAGE analysis highlighted infection by CEVd, CCaVd and another citrus viroid with a lower molecular weight than CEVd and belonging to group III of the citrus viroid catalog (4).

The trees derived from the budwood treated with gamma rays showed genetic mutations and produced fruits containing a low number of seeds. After biological and electrophoretic indexing, nearly all of the gamma ray treated plants showed the same virus and viroid infections as did the mother tree of Monreal Clementine, with the exception that the CCaVd and CVd-IIId had disappeared from budwood exposed to 4 and 6 kR (Fig. 1). However, there was no profile modifications seen in the budwood exposed to 2 kR. CPs-AV, CtRSV and all the viroids were eliminated by STG. Budwood treated with 8 and 10 kR were dried up by these higher amounts of radiation and died shortly after grafting. Ieki and Yamaguchi (6) eliminated CTV from infected budwood in Japan but CTV could not be tested for under our conditions in Italy since it has not been found here.

The results obtained are of great agronomic and phytosanitary interest. Clones obtained by gamma ray radiation producing very good fruits with few seeds and were productive in areas where Comune Clementine did not produce well. The general productivity of irradiated clones did not change in comparison with the

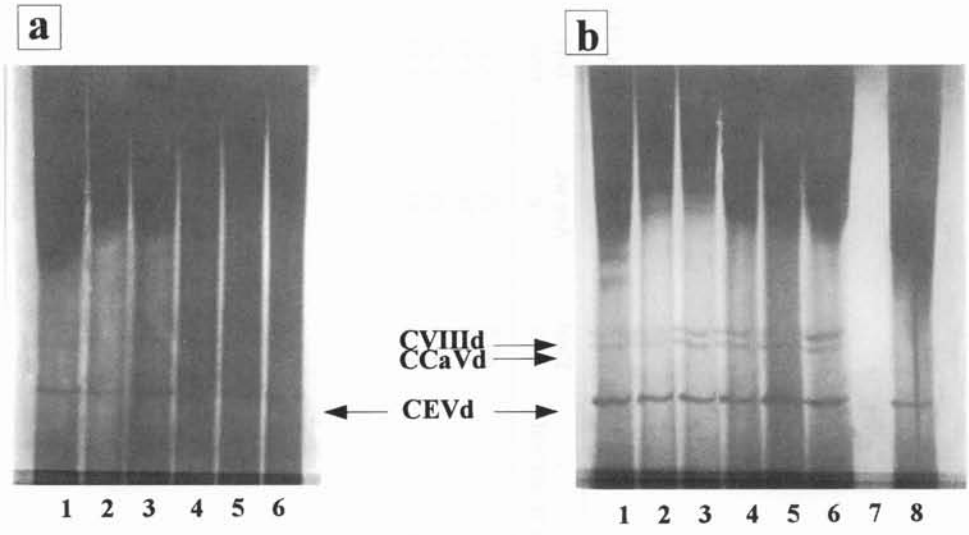

Fig. 1. dPAGE analysis of mother trees and plants obtained after irradiation with gamma rays; a) lanes 1-3: trees obtained from budwoods treated with 6 KR; lanes 4-6: trees obtained from budwoods treated with 4 KR; b) lanes 1-3: trees obtained from budwoods treated with 2 KR; lanes 4-6: mother trees; lane 7: empty; lane 8: CEVd marker.

TABLE 1
YIELD PER TREE AND FRUIT CHARACTERISTICS OF IRRADIATED AND UNTREATED CLEMENTINE CV. MONREAL

Gamma ray treatment	Yield (kg)	Avg wt. (g)	D/H ratio	Rind thickness (mm)	Juice content %	TSS %	Acids %	TSS/TA ratio	Avg no. of seeds
2kR	45.5	79	1.21	3.4	39.0	8.1	0.64	12.6	1.97
4kR	49.9	70	1.19	3.7	41.0	9.1	0.71	11.2	2.78
6kR	30.6	79	1.20	3.8	34.0	8.3	0.61	11.4	3.23
Untreated	41.5	81	1.13	4.3	36.0	9.0	0.73	12.3	20.00

old-line Monreal Clementine. Also, the organoleptic characteristics did not differ significantly from the old line except for the significant reduced number of seeds as shown in Table 1. Furthermore, the virus-free material obtained by STG is currently suitable to be propagated on rootstocks other than sour orange. These results allowed us to introduce these new gamma ray-treated lines in the Italian Voluntary Certification Program. The combined techniques of gamma-ray treatment and STG can be used with other citrus of commercial interest which are currently not propagated because they have characters not accepted by the consumers.

LITERATURE CITED

1. Albanese, G., R. La Rosa, and G. Polizzi
   1985. Indexing of citrus exocortis by bidirectional poly acrylamide gel electrophoresis analysis, p. 323-328. *In*: Proc. Integrated Pest Control in Citrus Groves, Acireale 26-29 Marzo, 1985.
2. de Lange, J. H.
   1978. Shoot tip grafting - a modified procedure. Citrus Subtrop. Fruit J. 539: 13-15.
3. Davino, M., G. Cartia, and A. Catara
   1983. La selezione sanitaria degli agrumi. Risultati acquisiti in Italia e prospettive, p. 333-350. *In*: Atti Convegno Internazionale su 'Evoluzione del quadro varietale ed organizzazione della ricerca nell'agrumicoltura mediterranea'. Catania, 22-23 Febbraio 1983.
4. Duran-Vila, N., C. N. Roistacher, R. Rivera Bustamante, and J. S. Semancik
   1988. A definition of citrus viroid groups and their relation to the exocortis disease. J. Gen. Virol. 69: 3039-3080.
5. Flores, R. and G. Llacer
   1988. Isolation of a viroid-like RNA associated with peach latent mosaic disease. Acta Horticult. 235: 325-332
6. Ieki, H. and A. Yamaguchi
   1984. Inactivation of citrus tristeza virus by gamma ray irradiation. Ann. Phytopathol. Soc. Jap. 50: 656-658.
7. La Rosa, R., G. Albanese, A. Azzaro, F. Sesto, and F. Domina
   1988. Suitability of nucleic acid analysis to diagnose viroid infections in citrus, p. 181-191. *In*: Proc. 10th Conf. IOCV., IOCV, Riverside.
8. Navarro, L., C. N. Roistacher, and T. Murashige
   1975. Improvement of shoot tip grafting *in vitro* for virus-free citrus. J. Amer. Soc. Hort. Sci. 100: 471-479.
9. Roistacher, C. N.
   1991. Graft-transmissible diseases of citrus. Handbook for Detection and Diagnosis. Publ. Div. FAO, Rome, 286 pp.
10. Russo, F., B. Donini, and A. Starrantino
    1981. Mutagenesis applied for citrus improvement. Proc. Int. Soc. Citriculture 1: 68-70.
11. Starrantino, A., P. Mannino, and F. Russo
    1988. The genetic stability of three seedless clonal selections obtained by gamma rays of the seedy 'Monreal' Clementine. Proc. Int. Soc. Citriculture 1: 77-84.
12. Starrantino, A.
    1991. Il microinnesto "in vitro" degli agrumi. Petria 2(1): 27-35.
13. Terranova, G. and F. Russo
    1968. The citrus budwood indexing of the Citrus Experiment Station of Acireale, p. 392-394. *In*: Proc. 4th Conf. IOCV. Univ. Florida Press, Gainesville.