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LETTER TO THE EDITOR

Eradication trials of tobacco mosaic virus using chemical drugs

A. LUVISI, A. PANATTONI, E. TRIOLO

Department of Tree Science, Entomology and Plant Pathology "G. Scaramuzzi", University of Pisa, Via del Borghetto 80, 56124 Pisa, Italy

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The science of virology was begun with research on tobacco mosaic virus (TMV) and a number of fundamental achievements in research in virology were obtained through the investigation of this virus (*1*). This virus was also used in chemotherapy trials as the main target of sanitation programs, even though it did not represent a harmful virus for most propagated plants. Beginning with the first studies in the 1960s, the eradication of TMV from plants seems to have presented a difficult challenge, as reported by Kurtzman *et al.* (*2*), in tobacco tissue culture not sanitized by various drugs, as well as in more recent studies on thiopurine administration in *in vitro* explants (*3*). Although the ability of plant compounds such as flavonoids to inhibit TMV infection appears well established (*4*), its resistance to eradication from plants using chemotherapy, compared to many other plant viruses, is unclear. TMV activity seems to be affected by 2,4-dioxo-hexahydro-1,3,5-triazine (*5*), dihydroxypropyladenine (*6*), nucleobase or nucleoside analogues (*7*), bitriazolyl compounds (*8*), tylophorine B (*9*), derivatives of thiadiazoleacetamide (*10*), as well as ribavirin in TMV-infected tobacco callus (*11*). From medical research, the inosine monophosphate dehydrogenase (IMPDH) inhibitors represent the most frequently used drugs able to eradicate viruses belonging to many genera, e.g. Capillovirus (*12*), Carlavirus (*13*), Caulimovirus (*14*), Closterovirus (*15*), Comovirus (*16*), Cucumovirus (*3*), Hordeivirus (*17*), Idaeovirus (*18*), Ilarvirus (*19*), Luteovirus (*20*), Nepovirus (*21*), Oryzavirus (*22*), Potexvirus (*23*), Potyvirus (*20*), Trichovirus (*24*), Vitivirus (*15*), including a Tobamovirus such as odontoglossum ringspot tobamovirus (*25*), suggesting that the activity of many antiviral drugs is not virus-specific. Each virus is characterized by specific strategies of replication in the cell, and the progress in the knowledge of the genome of many viruses and related enzyme requirements in the cellular metabolism has made it possible to identify other enzymes that can be used as a target for drugs. The final effect of their administration, however, depends not only on their mechanism of action, but also on the properties of the virus. Referring to the TMV resistance, antiviral drug mechanism can significantly interfere with many virus properties affecting virus turnover, such as virus longevity (*26*). Indeed, many viruses that can be eradicated by chemicals are characterized by *in vitro* longevity, which can range from a few hours to 150 days, as compared to 3.000 days for TMV (*27*).

We report the effect of ten drugs or pro-drugs commonly effective against various plant viruses on TMV eradication. Additional experiments to examine combined drug administration, long-lasting treatment and virus longevity were also performed to clarify the TMV resistance to chemical drugs. A drug-sensitive virus, cucumber mosaic virus (CMV), was selected to compare drug effectiveness as

E-mail: aluvisi@agr.unipi.it; phone: +39-050-2210560.

Abbreviations: 3-DG = deazaguanine; 6-TG = 6-thioguanine; BR = benzamide riboside; CMV = cucumber mosaic virus; HC = untreated negative control; IC = untreated positive control; IMPDH = inosine monophosphate dehydrogenase; DHPA = dihydroxypropyladenine; LCYS = L-cysteine ethyl ester, S-(N-methylcarbamate) mono chloride; LIV = *in vitro* longevity; MPA = mycophenolic acid; OS = oseltamivir; PCA = principal component analysis; RB = ribavirin; SA = selenazole; TIP = thermal inactivation point; TMV = tobacco mosaic virus; TR = tiazofurin

suggested by a number of chemotherapy successes in different plant species (*28*, *29*). For chemotherapy trials, *in vitro* tobacco explants were obtained from *Nicotiana tabacum* L. cv. Turkish, artificially infected by TMV or CMV, while healthy plants were used as control following D'Anna (*30*) protocols. IMPDH inhibitors (ribavirin, RB; tiazofurin, TR; selenazole, SA; benzamide riboside, BR; mycophenolic acid, MPA, kindly provided by Prof. Jayaram, Indiana University School of Medicine, Indianapolis, Indiana), neuraminidase inhibitor (oseltamivir, OS, Roche, Milan, Italy), S-adenosylhomocysteine hydrolase inhibitor (dihydroxypropyladenine, DHPA, kindly provided by Prof. Holy, Academy of Sciences of the Czech Republic, Prague, Czech Republic), purine biosynthesis inhibitors (6-thioguanine, 6-TG; deazaguanine, 3-DG; L-cysteine ethyl ester, S-(N-methylcarbamate) mono chloride, LCYS, kindly provided by Prof. Jayaram) were tested separately. Drugs were hydrated, ultra-filtered and added to proliferation medium after sterilization. A screening on healthy tobacco explants subjected to several drug concentrations (0.00, 0.10, 0.20, 0.30, 0.40 mmol/l) for six repeated subcultures was carried out to determine drug-induced phytotoxicity. The phytotoxic threshold was set at 30% dead explants as an acceptable mortality rate. Chemotherapy treatments involved drug administration for six consecutive subcultures, for a total treatment time of 90 days. Moreover, RB, TR and 6-TG were combined at a higher, non-phytotoxic concentration for a six-subculture-treatment against TMV to evaluate virus response to multiple drugs. Finally, a low dosage of TR (50% of maximum non-phytotoxic concentration) was administered to TMV-infected explants for long-lasting chemotherapy, with 18-subculture treatments, to evaluate the long-term virus response to the drug. After each treatment, the apical portion (1–2 cm) of each explant was transferred to fresh supplemented medium and the residue was assayed by ELISA (*31*). Polyclonal antibodies to TMV and CMV (Loewe Biochemica, Germany) were used and tissue samples from healthy (HC) and infected explants (IC) were used as negative and positive controls, respectively. Readings were normalized as R-values (OD-treated explant/OD-HC); R = 2.0 was used as a threshold to distinguish a positive versus a negative response (*32*). ELISA-negative explants were assayed by RT-PCR according to Eybishtz *et al.* (*33*) for TMV and Bertolini *et al.* (*34*) for CMV. RT-PCR was repeated after six and 12 months. All experiments were performed in triplicate; each experiment consisted of 15 explants infected with each virus.

In vitro longevity (LIV) was expressed as the time (days) that TMV and CMV remained infectious in crude juice kept at 20°C for 0, 1, 3, 6, 9, and 18 days (*35*). Test plants (*Chenopodium amaranticolor* for CMV and *N. tabacum* for TMV) were mechanically inoculated on six half-leaves of six plants per time per virus. After three weeks, the number of lesions per cm^2, which developed on inoculated leaves, was scored. Virus LIVs were measured also from *in vivo* plants, following the previously described method for LIV in crude juice.

The effects of treatments and differences in LIV were compared by analysis of variance in a random design using

Table. Percentage of ELISA-negative explants out of the total number of tobacco mosaic virus- or cucumber mosaic virus-infected *N. tabacum* cv. Xanthi explants assayed at the end of each subculture (15 days) treated with drugs

	ELISA-negative explants (%)										
	Tobacco mosaic virus-infected tobacco explants										
Subcultures	IC	RB	TR	6-TG	OS	DHPA	SA	BR	MPA	3-DG	LCYS
I	0.0a*	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
II	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
III	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
IV	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
V	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
VI	0.0a	2.9b	3.0b	2.9b	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
	Cucumber mosaic virus-infected tobacco explants										
Subcultures	IC	RB	TR	6-TG	OS	DHPA	SA	BR	MPA	3-DG	LCYS
I	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
II	0.0a	0.0a	4.4b	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
III	0.0a	0.0a	25.0c	11.4b	11.1b	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
IV	0.0a	0.0a	51.4d	30.8c	33.3c	0.0a	25.0c	7.5b	6.7b	0.0a	0.0a
V	0.0a	11.1b	64.7e	40.0d	40.0d	11.1b	40.5d	22.9c	22.5c	0.0a	0.0a
VI	0.0a	11.4b	66.7e	41.2d	44.4d	13.3b	44.4d	30.3c	24.3c	0.0a	0.0a

*Within the homogenate of the infected plant, values in the same line followed by the same letter do not differ significantly according to Duncan's multiple range test (P = 0.05). IC = untreated positive control; RB = ribavirin; TR = tiazofurin; 6-TG = 6-thioguanine; OS = oseltamivir; DHPA = dihydroxypropyladenine; SA = selenazole; BR = benzamide riboside; MPA = mycophenolic acid; 3-DG = 3-deazaguanine; LCYS = L-cysteine ethyl ester, S-(N-methylcarbamate) mono chloride.

CoStat software (version 6.203, CoHort Software, Monterey, USA). Duncan's multiple range test (P = 0.05) was calculated to determine significant differences. Principal Component Analysis (PCA) was performed to evaluate the influence of virus variables in chemotherapy trials. Viruses reported in the literature on chemotherapy trials – successfully administered or not – were used for PCA. In addition to TMV and CMV, viruses included in this test were apple chlorotic leaf spot virus, apple stem grooving virus, bamboo mosaic virus, barley stripe mosaic virus, chrysanthemum B virus, cymbidium mosaic virus, citrus tristeza virus, grapevine fanleaf virus, lily symptomless virus, odontoglossum ringspot virus, papaya mosaic virus, peanut mottle virus, plum pox virus, potato leafroll virus, potato S virus, potato X virus, potato Y virus, prune dwarf virus, prunus necrotic ringspot virus, raspberry bushy dwarf virus, rice ragged stunt virus, tomato mosaic virus, and tulip breaking virus. For these viruses, the variables used in the test were: rate of eradication by chemicals, LIV, thermal inactivation point (TIP), size, genome partition, percentage of RNA (RNA %) and guanine-cytosine content (GC %), retrieved from the literature (*27*).

Considering phytotoxic screening, the dosages that caused a less than threshold toxic effect on healthy explants were up to 0.40 mmol/l for OS, DHPA, SA and MPA, up to 0.30 mmol/l for 6-TG and LCYS, up to 0.20 mmol/l for TR and 3-DG or up to 0.10 mmol/l for RB. Chemotherapy test showed that none of the TMV-infected explants treated with six out of nine drugs showed R-values below threshold. Treatments with RB, TR or 6-TG were effective against TMV and gave 2.9, 3.0 and 2.9% ELISA negative plantlets, respectively. However, all ELISA-negative explants produced amplicons of expected size after RT-PCR assay, meaning plant sanitation was not achieved. Unlike TMV, treatments against CMV led to better results. CMV-infected tobacco explants treated with 3-DG or LCYS did not present ELISA readings below the threshold value for negative response, whereas explants treated with other drugs showed R values below the threshold, with up to 66.7% of ELISA-negative plantlets for TR-treatment. Sanitation rates assessed by RT-PCR were 5.7% for RB, 44.4% for TR, 17.8% for 6-TG and SA, 15.5% for OS, 6.7% for DHPA, 4.4% for BR and 8.9% for MPA. These results were confirmed by RT-PCR after six and 12 months.

Combined drug administration did not increase the number of explants below ELISA threshold after six subcultures. Conversely, long-lasting treatments with TR (18 subcultures, 270 days) increased the ELISA-negative explants up to 11.8 %. However, RT-PCR test did not confirm the eradication of TMV.

Considering virus LIV at the lower drug concentration, the infectivity of TMV was not reduced during the test period, while at the higher concentration a decrease in infectivity was reported after nine days. TMV infectivity was maintained for 18 days after sap preparation. In contrast, CMV infectivity was significantly reduced after one day, and completely lost after six days. Virus source did not affect LIV. Pearson's correlation matrix calculated from data available from literature on plant chemotherapy showed that the virus eradication is negatively correlated to LIV and TIP. The genome partition and RNA % are positively correlated to eradication. Considering PCA performed on data available from the literature on the plant chemotherapy, the first principal component axis accounted for 35% of the observed variation. It was strongly negatively correlated with LIV and TIP, and positively correlated to the eradication, and it separated LIV and TIP from other parameters, as well as eradication. The second principal component axis was not substantially correlated with eradication. Considering the mechanism involved in the chemotherapy trial, where the temperature should not be an essential factor for drug efficacy, LIV remains one of the main candidates for interfering negatively with the chemotherapy results. Anyway, other parameters, such as the physiological and developmental stage of the host tissue, have effect on the efficacy of chemotherapy, quite possible differing between viruses because of their different mode of cell-to-cell and long-distance movement within the plant.

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