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Nonthermal Microwave Radiations Affect the Hypersensitive Response of Tobacco to Tobacco Mosaic Virus

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ABSTRACT

Objectives: The aim of the present study was to evaluate the effects of nonthermal extremely high-frequency microwave radiations in a plant-based bioassay, represented by tobacco plants reacting to tobacco mosaic virus with a hypersensitive response leading to the appearance of necrotic lesions at the infection sites.

Design: This study was performed blind and different experimental protocols on tobacco plants inoculated with tobacco mosaic virus were used.

Bio-objects: Tobacco plants (*Nicotiana tabacum* L. cultivar Samsun) carrying the resistance gene N against tobacco mosaic virus.

Interventions: Tobacco plants or leaf disks were either directly or indirectly (water-mediated) irradiated using a medical device, designed for microwave resonance therapy. It produces nonthermal weak-intensity extremely high-frequency radiations, either modulated at extremely low frequency or in continuous flux of waves, coupled with a nonthermal red/near-infrared radiation.

Outcome measurements: The working variable was the number of hypersensitive lesions per leaf disk.

Results: Both direct and indirect nonthermal microwave radiations led to significant effects on the hypersensitive response of tobacco plants: modulated radiations generally induced a resistance increase, whereas a continuous flux of waves induced a resistance decrease with direct treatments only.

Conclusions: Nonthermal microwave radiations are effective on the hypersensitive response of tobacco to tobacco mosaic virus and their low-frequency modulation seems to be more bioactive than the continuous-flux of waves, particularly in the indirect water-mediated treatments.

INTRODUCTION

Today the scientific community shares the opinion that electromagnetic fields (EMFs), as nonionizing radiations, contain photon energy large enough to cause various types of damage, ranging from the stretching to the breakdown of hydrogen bonds (Rai, 1997), by heating bio-objects. One of the first biologic mechanisms to be identified and confirmed is calcium ion efflux (Blackman, 1990). Calcium ions were induced to flow out of or into cells, depending on combination of exposure conditions. These combinations are known as "windows," because nearby conditions have markedly different effects (Blackman, 1989). Radiofrequency-induced Ca^{2+} efflux is associated with enhanced programmed cell death (PCD), whereas extremely low frequency (ELF)-induced Ca^{2+} influx is associated with

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enhanced cell survival of damaged cells (i.e., it enhances cancer; Bawin and Adey, 1976).

On the other hand, in the last few decades several researchers have outlined direct or water-mediated nonthermal effects of EMFs on free ions, on rate and direction of biochemical reactions (such as the melatonin/serotonin balance), and on cell replication (Adey, 1993; Cherry, 1998; Devyatkov, 1974; Rai et al., 1999; Sato et al., 1996; Singh et al., 1994; Webb, 1975). Recently, a nonthermal heat–shock response to microwaves has been reported: the hypothesized mechanism includes disruption of weak bonds of protein active forms, enhanced production of reactive oxygen species, and interference with cell-signaling pathways (De Pomerai et al., 2000).

Instrumental data confirm water EMF-induced structural changes and their persistence for extended periods of time (Del Giudice and Preparata, 1998; Fesenko and Gluvstein, 1995; Lobyshev et al., 1999; Preparata, 1995). Moreover, visualization modality such as electrography (also known as Kirlian photography) has shown a distinct and nonlinear behavior of the corona discharge in water drops exposed to different microwave power densities (Kmecl et al., 2000; Skarja et al., 1998); this technique may be perfectible by specifically calculating the optimal dimensions of the experimental exposure chambers (Alekseev and Ziskin, 2001).

Concerning microwave interaction with living systems, differential effects have been recently reviewed (Banik et al., 2003). In particular, nonthermal bioeffects have been studied on fungal spore germination after continuous-flux of waves (cw) or modulated microwave irradiation (Rai et al., 1994a, 1994b). Other experiments with microwave radiation have demonstrated an electrical effect on *Mimosa pudica* action potential (Spanoudaki et al., 1999), an increased growth of *Saccharomyces cerevisiae* and *Spirulina* sp. (Kositsky et al., 2001) and a stimulatory effect on pollen germination and pollen tube growth (Calzoni et al., 2003). Finally, modulated extremely high-frequency electromagnetic radiation of low intensity resulted in the activatation or inhibition of respiratory burst in neutrophils of the mice line NMRI, depending on modulation frequency (Gapeev et al., 1997).

The aim of the present work was to provide a further experimental contribution on the bioeffects of weak microwave radiation on a well-known phytopathologic "sensor" system: tobacco plants reacting to tobacco mosaic virus (TMV) with a hypersensitive response (HR). This defense mechanism consists of rapid cell necrosis at the infection site and is associated with restricted pathogen multiplication and spread (Kombrink and Schmelzer, 2001). The higher the resistance level, the quicker the defense response expressed by fewer and smaller necrotic lesions (Ross, 1961). HR includes transient changes in ion fluxes across the plasma membrane and intracellular signal transduction mechanisms that involve protein phosphorylation and reactive oxygen species accumulation (Numberger and Scheel, 2001; Seo et al., 2000). Several studies have shown striking homologies between HR cell death in plants and PCD in animals (Jabs, 1999; Lam et al., 2001), supporting the suggestive idea of a PCD shared mechanism in eukaryotics.

In recent years, many studies have focused on the effects of nonthermal microwaves on apoptosis, a form of animal PCD: an activation of apoptotic processes at microscopic-, mesoscopic- and macroscopic levels has been pointed out (Garaj-Vrhovac et al., 1990; Marinelli et al., 2004; Ye et al., 2001). These nonionizing millimetric frequencies would be reduced to micrometric and nanometric dimensions by electroacoustic transduction and thus adapted to enter in spatial resonance with the intracellular biostructures (Bistolfi, 1998; Golant, 1994). In such natural biocybernetic systems, actually the extremely high-frequency (EHF) range could play a function of medium frequency allowing the correlation of fields within an organism and the transfer of information by continuous or modulated flux (Afromeev et al., 1997). Concerning the weak (up to 20 mW) red/near-infrared radiation (635–950 nm) emitted together with microwaves by the device utilized in our experiments, recent experiences with much more intense pulsed light have shown that only wavelengths longer than 950 nm would lead to nonspecific heating of tissue water (Bjerring et al., 2001).

Plant-based models, lacking placebo effect and allowing a high number of replications (Betti et al., 2003), are useful for studying the nonlinear responses typical of nonthermal electromagnetic radiations (Fröhlich, 1982) and the connected problem of irreproducibility (Chukova, 1999).

MATERIALS AND METHODS

Plants and virus

Plants of *Nicotiana tabacum* L. cultivar Samsun, carrying the TMV resistance gene N (Marathe et al., 2002) were grown in a greenhouse under controlled conditions (16-hour/8-hour day/night cycle, 25 ± 1°C, 1.87 W m^{-2} intensity light, 75%–80% relative humidity) up to the vegetative stage of approximately nine mature leaves.

Highly purified TMV type-strain suspension was obtained as previously described (Betti et al., 1997; Fraser and Gerwitz, 1985). The virus inoculation was performed by uniformly dropping and gently rubbing the adaxial leaf surface with 200 μL per leaf of the TMV suspension (20 μg ml^{-1} in 10 mmol/L $NaKPO_4$ pH 7.0), added to 0.1 mg mL^{-1} carborundum 400 mesh size as abrasive. After inoculation, the leaf was rinsed with tap water. The working variable was the number of hypersensitive lesions per disk evaluated at the third day from virus inoculation.

Electromagnetic treatment

The irradiation source was a medical device designed for microwave resonance therapy. It produces a nonthermal weak intensity microwave beam characterized by a power density (PD) of 10^{-12} Wcm^{-2} and by an EHF wide-band radiation from 40 to 78 GHz, either in continuous-flux of waves (cw) or modulated in frequency at 10, 100, and 1000 Hz. This radiation is coupled with another nonthermal broad-band radiation generated by an optoelectronic light emitting diode (LED) of low intensity (up to 20 mW) ranging from visible light to near-infrared (λ from 635 to 950 nm).

To assess the basic EMF experimental conditions, measurements of the electric and magnetic field in the laboratory and glasshouse environment were performed by a PMM 8053 (EMF measuring unit; PMM-MPB, Rome, Italy) equipped with PMM EHP-50 probe. The basic experimental conditions are reported in Table 1.

Leaf disk and water irradiation

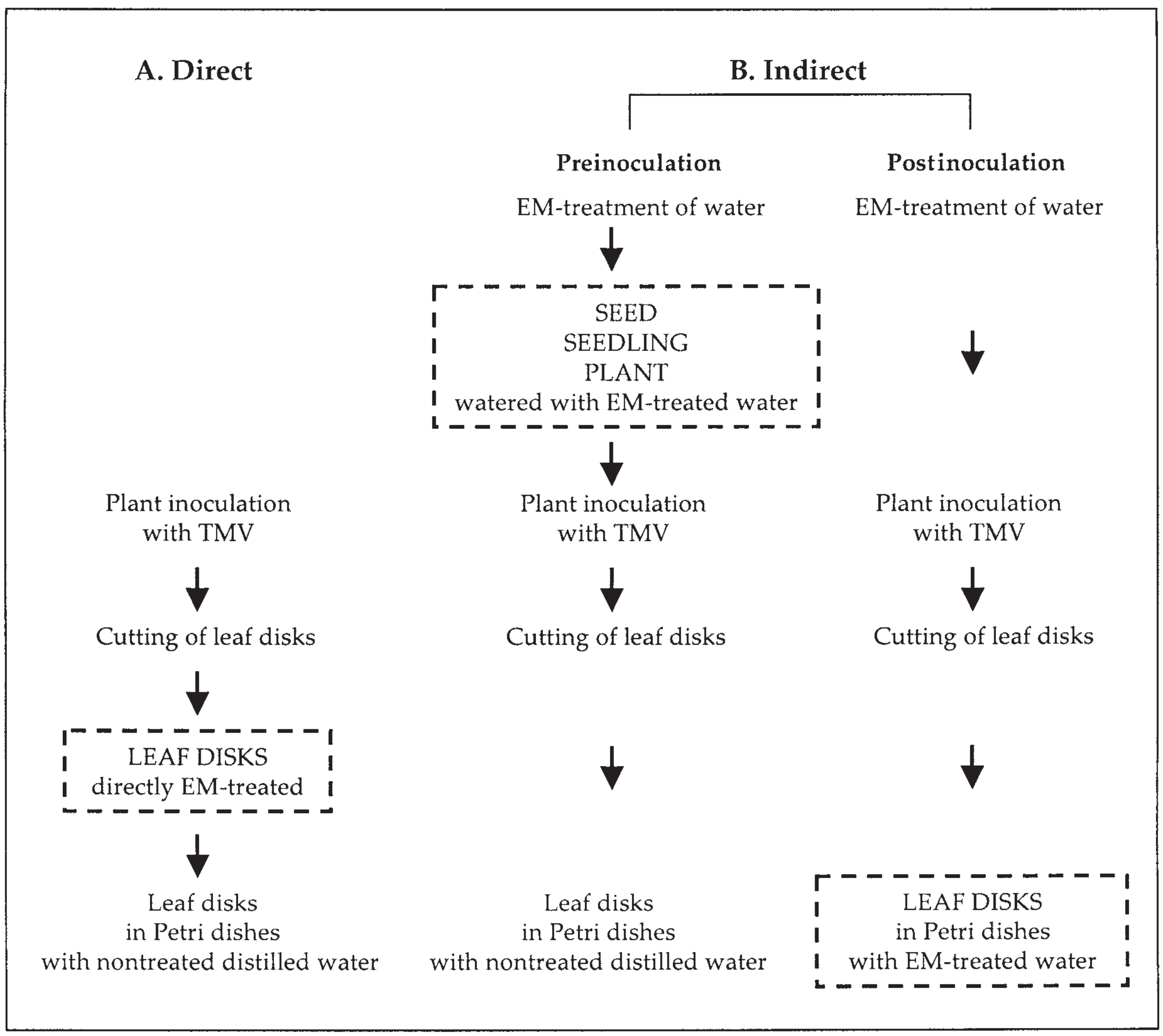
Leaf disks (20 mm diameter), cut from inoculated leaves and placed on humid filter papers, underwent to the treatment, 1 cm from the irradiator (Control disks did not undergo treatment.)

TABLE 1. BASIC EXPERIMENTAL CONDITIONS OF THE 50-HZ MAGNETIC AND ELECTRIC FIELD IN CONTROL SAMPLE PLACES (CP) AND IRRADIATED SAMPLE PLACES (IP), IN LABORATORY AND GREENHOUSE

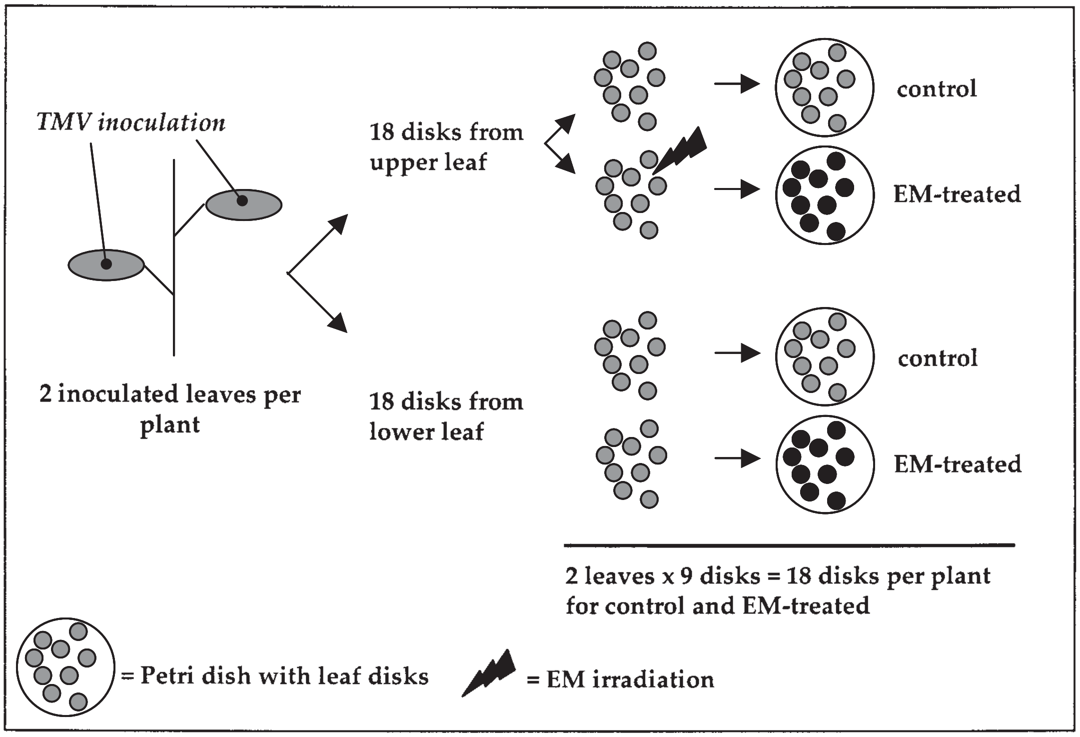
	Magnetic field (μT)		*Electric field (Vm^{-1})*	
Measurement place	*CP*	*IP*	*CP*	*IP*
Laboratory	0.014	0.013	101.2	101.3
Greenhouse	0.060	0.065	21.9	21.6

The measurements in the laboratory were made upon the exposed samples in the place where irradiation was performed, 1 m from the power supply.

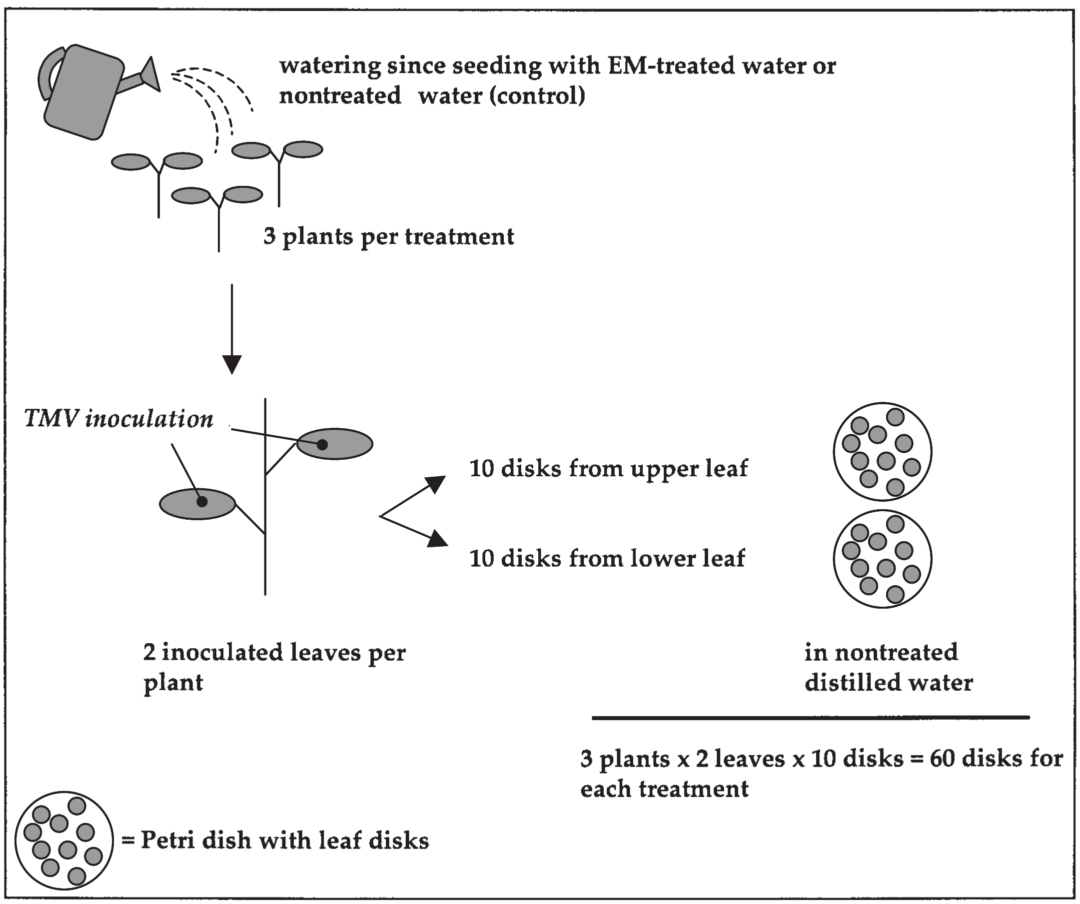
Standard volumes of distilled water (1.5 μS cm^{-1}, pH 5.5) in Pyrex beakers underwent treatment (controls did not) by placing the irradiator on the liquid surface. The samples were prepared 24 hours before use and stored at room tem-



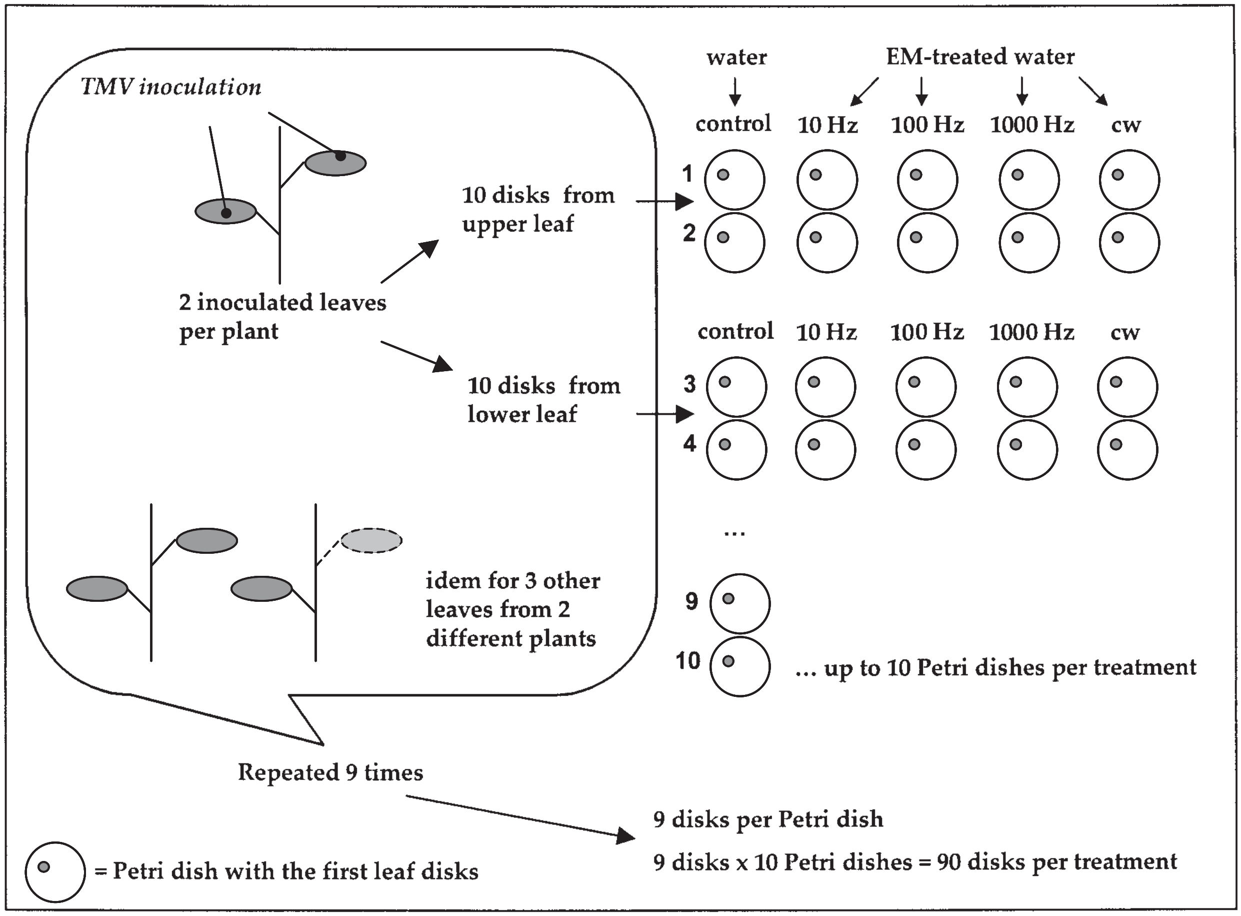
SCHEME 1. Experimental steps of direct (A) and indirect (B) treatments. Dotted boxes show when bioelectromagnetic interaction occurs. EM, electromagnetic; TMV, tobacco mosaic virus.



SCHEME 2. Experimental protocol for direct treatment. TMV, tobacco mosaic virus; EM, electromagnetic.



SCHEME 3. Experimental protocol for indirect pre-inoculation treatment. EM, electromagnetic; TMV, tobacco mosaic virus.



SCHEME 4. Experimental protocol for indirect post-inoculation treatment. TMV, tobacco mosaic virus; EM, electromagnetic; CW, continuous-flux of waves.

perature. In water-mediated preinoculation experiments the same procedure was adopted using tap water.

Experimental set-up

The steps of direct and indirect treatments are summarized in Scheme 1, where it is also evidenced when the bio-electromagnetic interaction occurs. In direct treatments leaf samples were directly exposed to electromagnetic (EM) irradiation; in indirect ones leaf samples or plants were treated by EM-irradiated water. All the experiments were performed using a blinded protocol.

Direct treatment. As shown in Scheme 2, 2 leaves per plant, the third and the fourth from the top (upper and lower, respectively), were inoculated with TMV; 18 disks per each leaf were cut and randomly divided into 2 groups: 1 was exposed to EM irradiation (EM-treated) and the other, not EM-irradiated, was the control. The disks (both EM-treated and control) were then placed in Petri dishes, each containing 15 mL of nontreated distilled water. Using 2 leaves per plant, a total of 18 disks per treatment were obtained. Five (5) plants per treatment (from 40 to 78 GHz modulated at 10, 100, and 1000 Hz and cw) were used.

Indirect treatment. The EM-treated water was used (1) before TMV inoculation (preinoculation experiments) or (2) after TMV inoculation (postinoculation experiments). All the trials were repeated three times both in preinoculation and postinoculation experiments.

As shown in Scheme 3, in preinoculation experiments tobacco plants were watered (from seeding until 9-leaf stage) with EM-treated water or nontreated water (control). Two leaves per plant, the third and the fourth from the top (upper and lower, respectively), were inoculated with TMV; 10 disks per each leaf were cut and arranged in a petri dish containing 15 mL of nontreated distilled water. Using 3 plants per treatment (water irradiated in the range of 40 to 78 GHz modulated at 10Hz and cw, or not-irradiated for the control), 60 disks per treatment were obtained.

As shown in Scheme 4, 2 leaves per plant, the third and the fourth from the top (upper and lower, respectively), were inoculated with TMV; 10 disks were cut from upper leaf and arranged one by one in 10 petri dishes (2 dishes per treatment) containing 15 mL of the different treatments (water irradiated in the range of 40 to 78 GHz modulated at 10, 100, and 1000 Hz and cw, or not-irradiated for the control). This procedure was repeated for the lower leaf arranging the disks in other 10 petri dishes and for other 3 leaves (from 2

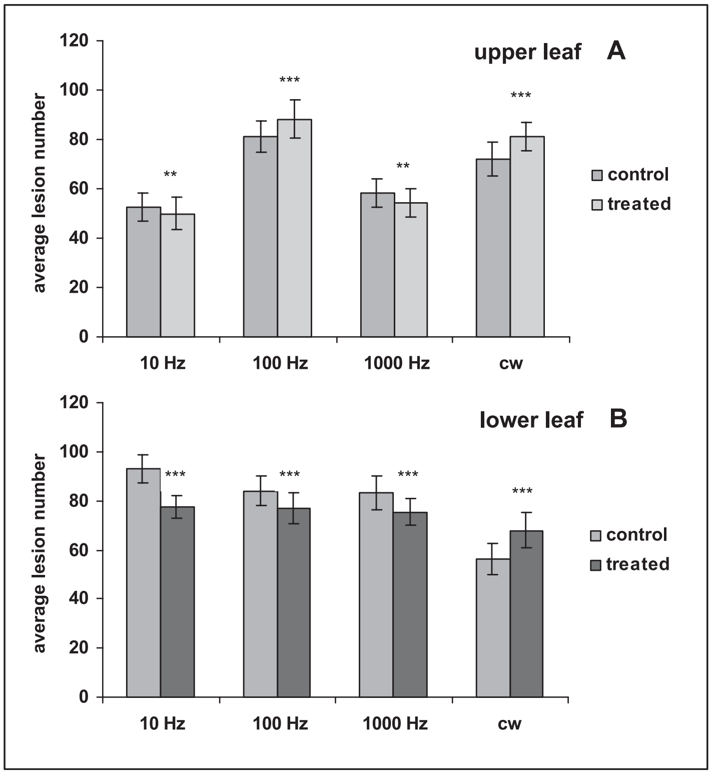


FIG. 1. Direct treatments: Average number of hypersensitive lesions for each treatment in upper (**A**) and lower (**B**) leaves. Each bar represents the mean of 45 disks from 5 leaves of different plants. Statistical significance given by Wilcoxon test (*** = $p < 0.001$; ** = $p < 0.01$). cw, continuous-flux of waves.

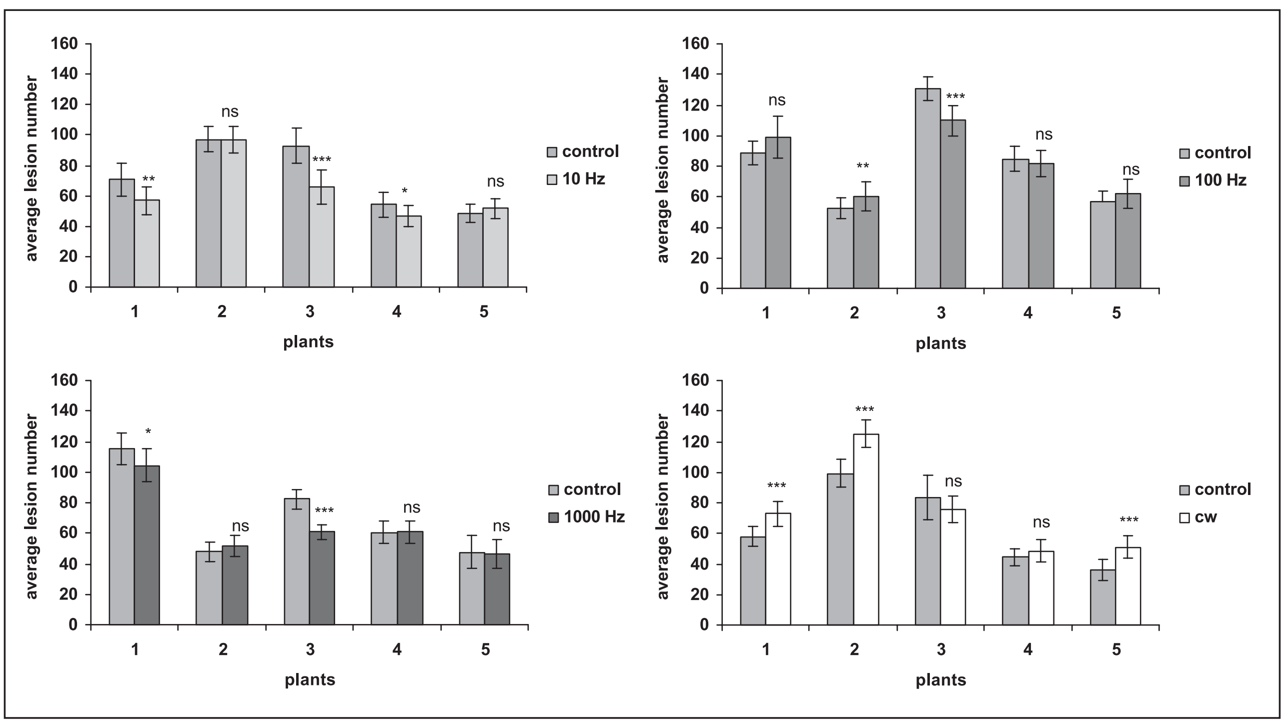


FIG. 2. Direct treatments: Average number of hypersensitive lesions for each treatment in single plants. Each bar represents the mean of 18 disks from upper and lower leaves of each plant. Statistical significance given by Wilcoxon test (*** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$; ns = not significant). cw, continuous-flux of waves.

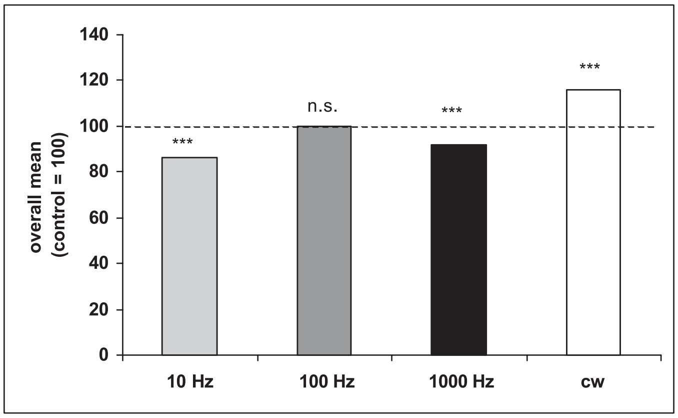


FIG. 3. Direct treatments: Overall mean of hypersensitive lesions with respect to control (= 100). Each bar represents the joint mean of 90 disks from 5 plants per treatment. Statistical significance given by Wilcoxon test (*** = $p < 0.001$; ns, not significant). cw, continuous-flux of waves.

different plants), so that one disk was placed in each of the 10 dishes containing the same treatment. More disks were then added up to 9 disks per petri dish, following the same protocol and using a total of 45 leaves from 23 plants. In this way 90 disks per treatment were obtained and 2 disks from each leaf were present in each treatment.

Lesion counting

Digital images were recorded by scanning leaf disks with a flatbed scanner (Hewlett-Packard Scanjet 5470c; Hewlett-Packard, Palo Alto, CA) at 600 dots per inch. Purpose-developed software allowed lesion recognition and counting.

Statistical analysis

In all the experiments performed the leaf disk is the elementary statistical unit and the working variable Y is the hypersensitive lesion number per disk. We have considered the leaf disks separately, after checking previously that variability "within" (disks from the same leaf) is higher than variability "between" (leaves from different plants), both for upper and lower leaf. In indirect postinoculation experiments, in which the experimental protocol adopted allowed a randomization, the samples had been randomized and built through a stratified sampling procedure, where each plant (*stratum*) were represented in each treatment group.

Some exploratory statistics were computed, such as mean M(Y), standard deviation S(Y), median Me(Y), and mean absolute deviation about the median (MAD). Because the data were sensibly skewed, we decided to apply the nonparametric Wilcoxon rank sum test. The test statistic involves not only the median values, but all the sample observations, therefore, it may be significant even if sample medians are not sensibly different (or vice versa). Because we were dealing with a multiple comparison, the significance was corrected by Bonferroni adjustment, in order to reduce the probability of a false significance (Galambos and Simonelli, 1996). Finally, in water-mediated experiments, treatment groups were compared with control by Student's *t* test, considering that overall samples are large enough.

RESULTS

To check the external or spurious EMF presence, the basic 50-Hz electric and magnetic fields were measured (Table 1). No basic differences were detected between control and exposure place.

Direct treatment

The results obtained by direct irradiation of inoculated leaf disks are reported in Figures 1, 2, and 3. For each treatment, we noticed that lower leaves generally present more lesions than upper leaves (Fig. 1A and 1B); nevertheless, the direction of the treatment effect (always highly significant) is the same for upper and lower leaves, except for 100-Hz modulated treatment that induced opposite effects. Considering for each treatment the average number of hypersensitive lesions per each plant (Fig. 2), we observed a trend toward a de-

TABLE 2. INDIRECT PREINOCULATION TREATMENTS: EFFECT OF WEAK EHF MWS (MODULATED AT 10 HZ AND CW) ON THE DISTRIBUTION OF HYPERSENSITIVE LESIONS

Experiments[a]	*Statistics*		*Control*		*10 Hz*		*cw*	
I	M(Y)	S(Y)	73.4	40.7	33.9	29.0	93.0	42.8
	Me	MAD	73.0	34.2	30.5	21.2	93.5	32.0
II	M(Y)	S(Y)	70.2	35.6	49.4	39.9	32.1	29.1
	Me	MAD	71.0	28.7	43.5	34.6	26.5	20.2
III	M(Y)	S(Y)	32.6	21.9	42.8	30.0	54.1	32.2
	Me	MAD	18.0	26.1	42.5	25.6	49.0	27.8

[a]Experiments have been labeled by Roman numerals.
$n = 90$ data per treatment and experiment.
M(Y), average number of lesions, S(Y), standard deviation; Me, median; MAD, mean absolute deviation about the median.
EHF, extremely high frequency; MWs, microwaves; cw, continuous wave.

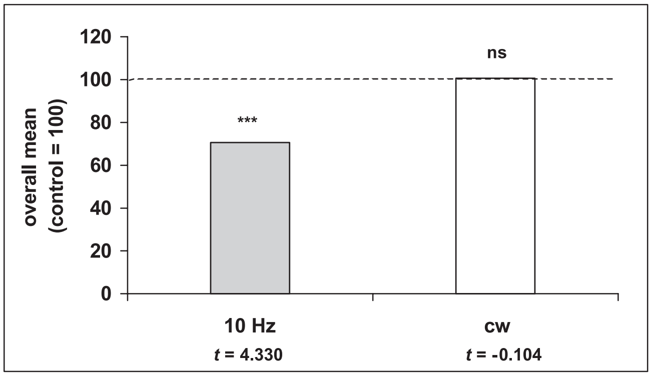


FIG. 4. Indirect preinoculation treatments: Overall mean of hypersensitive lesions for each treatment. Each bar represents the mean of three experiments. Statistical significance given by Student's *t* test: *** ($p < 0.001$); ns (not significant). cw, continuous-flux of waves.

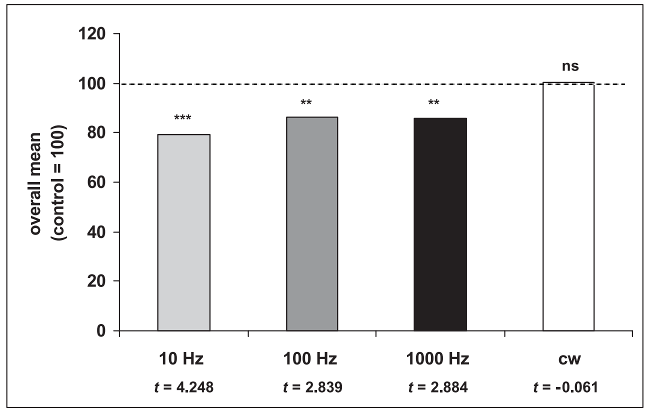


FIG. 5. Indirect postinoculation treatments: Overall mean of hypersensitive lesions for each treatment. Each bar represents the mean of three experiments (n = 270). Statistical significance given by Student's *t* test: *** ($p < 0.001$); ** ($p < 0.01$); ns (not significant). cw, continuous-flux of waves.

crease or an increase of lesion number following on 10-Hz modulated and cw treatments, respectively. Finally, in Figure 3 the overall mean of hypersensitive lesions is reported: 10- and 1000-Hz modulated treatments induced a significant decrease versus control, whereas cw treatment led to a significant increase.

Indirect treatment

Preinoculation experiments. Table 2 shows the effects of 10-Hz modulated and cw treatments: depending on the experiment, each treatment induced a sensible modification in average lesion number with a decreasing or increasing trend for 10-Hz modulated and cw treatments, respectively. The overall mean of hypersensitive lesion number for each treatment is presented in Figure 4: 10-Hz modulated treatment induced a significant decrease in lesion number, whereas cw treatment did not show significant results.

Postinoculation experiments. The results obtained in three successive experiments with EM treatments are reported in Table 3. Depending on the experiment, each treatment induced a sensible modification in average lesion number. A general decreasing trend was observed, in particular for 1000 Hz modulated treatment. The overall mean of hypersensitive lesion number for each treatment is reported in Figure 5: modulated treatments induced a highly significant lesion number decrease versus controls, whereas cw treatment did not show any significant result.

Overall analysis

The effects of direct and indirect irradiation are compared in Figure 6, by means of the average number of lesions. The 10-Hz modulated and cw treatments were tested in indirect (preinoculation and postinoculation) and direct experiments: specifically, 10-Hz modulation induced a systematic decrease in lesion number (resistance increase) for every experimental setting, whereas cw (not significant with indirect treatments) induced a significant increase in lesion number (resistance decrease) with direct irradiation. Moreover, it can be seen that all modulated treatments show a general trend toward a lesion number decrease.

TABLE 3. INDIRECT POSTINOCULATION TREATMENTS: EFFECT OF WEAK EHF MWS (MODULATED AT 10, 100, 1000 HZ AND CW) ON THE DISTRIBUTION OF HYPERSENSITIVE LESIONS

Experiments[a]	*Statistics*		*Control*		*10 Hz*		*100 Hz*		*1000 Hz*		*cw*	
I	M(Y)	S(Y)	73.5	41.6	55.2	38.6	62.8	33.4	65.5	42.3	82.8	52.1
	Me	MAD	63.5	31.6	50.0	30.3	60.0	26.3	60.0	30.5	78.0	33.4
II	M(Y)	S(Y)	62.9	29.7	81.7	38.7	63.8	37.2	57.2	35.2	68.3	37.7
	Me	MAD	67.0	24.4	80.5	31.8	57.5	31.1	53.0	25.8	61.5	30.1
III	M(Y)	S(Y)	88.3	49.1	41.0	41.2	67.4	51.8	70.5	47.1	74.3	47.0
	Me	MAD	86.5	37.0	28.0	30.9	64.0	42.0	63.0	38.5	70.0	37.9

[a]Experiments have been labeled by Roman numerals.
$n = 90$ data per treatment and experiment.
M(Y), average number of lesions; S(Y), standard deviation; Me, median; MAD, mean absolute deviation about the median; EHF, extremely high frequency; MWs, microwaves; cw, continuous wave.

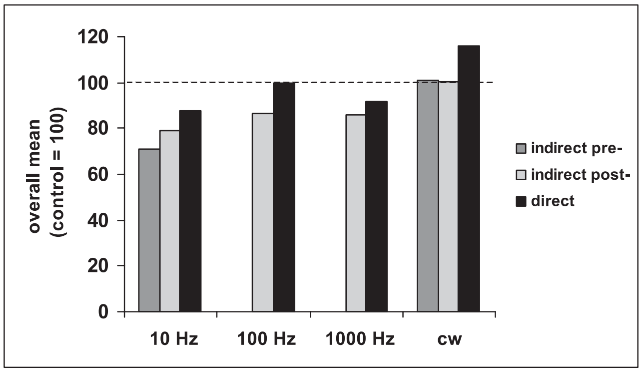


FIG. 6. Effects on the average lesion number of indirect (pre- and postinoculation) and direct irradiation. cw, continuous-flux of waves.

DISCUSSION

Our results show that nonthermal microwave radiations induce significant effects on the tobacco/TMV model. Our findings point out that ELF-modulated treatments generally induce a significant increase of resistance. In particular, it is noteworthy that 10-Hz–modulated treatment induced a resistance increase for every experimental setting, providing more evident results when plants are treated with irradiated water from seeding (indirect preinoculation experiments). This finding suggests that the resistance improvement of plant to virus (probably the result of a reinforcement of the natural PCD) could be ascribed to a prolonged interaction between irradiated water and bio-object. Moreover, the efficacy observed in indirect experiments seems to imply that the structural changes in EM-induced water are long lasting, as supposed and evidenced by physical and chemical researches (Del Giudice and Preparata, 1998; Elia and Piccoli, 1999; Fesenko and Gluvstein, 1995; Lobyshev et al., 1999; Preparata, 1995; Rai et al., 1995). According to the water structure models (Franks, 1972; Neilsons and Enderly, 1986) and to the quantum electrodynamical (QED) coherence hypothesis (Del Giudice and Preparata, 1998), the water molecules can be found in a free state (bulk water) or organized in clusters where they oscillate in phase (bound water). It can be hypothesized that the effect on hypersensitive response we detected in the indirect experiments is correlated to water as a bound system, which could react to the ELF modulated irradiation.

Regarding the electromagnetic treatment without ELF modulation (continuous-flux of waves, cw), a resistance decrease (shown by a higher number of necrotic lesions resulting from a reduction of hypersensitive response) in direct treatment was observed. In agreement with the shared opinion on homology between hypersensitive response in plants and PCD in animals, this finding could be interpreted as an amplification of an anti-PCD trend. Therefore, hypothesizing an homeostatic function of PCD, our experimental results (stimulation or inhibition of plant PCD by means of modulated and continuous-flux of waves treatments, respectively) would justify the current clinical utilization of microwave resonance therapy either in auto-immune diseases and in cancer or in degenerative pathologies (Frey, 1993; Jovanovic-Ignjatic and Rakovic, 1999). On the other hand, the cw treatment did not induce significant results in indirect experiments. This finding and the significant results obtained with the same treatment but ELF modulated would confirm that the most active component of the electromagnetic signal, supplied to the bio-object through water mediation, could be the low frequencies (ELF), as hypothesised by others (Panogopoulos et al., 2002). In particular, the nonsignificant results of the cw indirect treatments may be ascribed to the extremely rapid changes (every 10^{-11} seconds) in the water hydrogen bonds (Cooke and Kuntz, 1974): this feature does not allow the water to react as a typical complex system.

CONCLUSIONS

Our macroscopic observations may be microscopically explained by the interaction between microwaves and the cytoskeleton network that represents both the origin of endogenous electromagnetic activity, in respect of Fröhlich's theory of coherent vibration states (Fröhlich, 1968), and the target for external irradiations (Jelinek and Pokorny, 2001; Pokorny, 1999), according to the frequency coupling theory of the temporal resonance (Illarionov, 1998) and to the electroacoustic transduction of the microspatial resonance theory (Bistolfi, 1998; Golant, 1994). The cytoskeleton has been proposed as a target for other kinds of exogenous irradiation in addition to microwaves: in particular, the microtubule cavities as individual components would selectively react to soft x-rays band, while the cytoskeleton as a whole system would respond to the far-infrared region (Jelinek and Pokorny, 2001; Pokorny, 1999). Because these effective bands are too much higher or lower than the red/near-infrared range, we could exclude that this component of our electromagnetic emission has acted on the cytoskeleton in the results we obtained.

Moreover other theoretical resonance models, including the cyclotronic (Liboff, 1985), the parametric (Lednev, 1991), the gyroscopic (Binhi, 2002; Binhi and Savin, 2002) and the forced coherent vibration models (Panagopoulos et al., 2002), would justify the capability of the nonthermal ELF-modulated microwave irradiation to convert the electromagnetic signal into a biochemical response, in spite of the thermal noise of the bio-object the energy scale of which, Kt, is several orders greater than the weak quantum energy irradiated. The DNA itself would be involved in these interactions (Banik et al., 2003). Our basic research, sharing this background with so-called energy medicine, could offer a valid test-bed to investigate its still unsolved questions.

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