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Experimental Analysis of the Potential Validity of Lorenzini's Hypothesis to Treat COVID-19 Patients

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ABSTRACT

The present research was aimed at testing the potential validity of Lorenzini's hypothesis against Covid-19, based on the use of far UV-C rays directly on the patient's ill lungs, so to significantly reduce the virus presence and with it the interstitial pneumonia, the cause of most deaths. Not having had the chance to experiment directly on the SARS-CoV-2, we shifted to a plant virus called TSWV, which has some common characteristics with SARS-CoV-2, so making our tests significant. The virus was suitably utilized so to affect an opportune range of pepper plants, after a treatment with 2 UV-C lamps, of which just one spreading far UV-C rays. Results showed that the effect of a quick exposition to UV-C rays of TSWV-infected sap extract reduces nearly to zero the viral effect, leaving the plant healthy or infected, but asymptomatic. Due to the test conditions and to the similarities of TSWV to SARS-CoV-2, we can conclude that Lorenzini's hypothesis against Covid-19 is well posed and, if adopted, could save many lives.

1. INTRODUCTION

Recently it was hypothesized a therapy to treat Covid-19 patients [1], especially those in "phase 3" (the most dangerous condition), when the risk of Interstitial Pneumonia arises, causing in a significant range of cases the death of the patient by asphyxia. The hypothesis made was that a close application of far UV-C rays (wavelength between 207 and 222 nm) could significantly reduce the viral presence, so letting the patient breath almost normally. It has been hypothesized a cylindrical probe emitting far UV-C rays, inserted via intubation in the patient, and located as near as possible to the patient's lungs. The choice of far UV-C rays is due to the fact it was demonstrated that they are the ones less harmful to people [2]. To properly experiment on the SARS-CoV-2 virus we should have needed level 3 biosafety laboratory, but it was not possible to find one available throughout our Country. Thus, we adopted another strategy, to verify if Lorenzini's hypothesis [1] is well posed: we used a plant virus called Tomato spotted wilt virus (TSWV), which shows some common characteristics with SARS-CoV-2, but can be tested in an ordinary plant pathology laboratory. *Tomato spotted wilt virus* (TSWV) belongs to the family *Bunyaviridae*, genus *Tospovirus*. The family *Bunyaviridae* includes more than 300 species of viruses, and their genome is composed of single-stranded RNA (ssRNA), and they infect plants, insects and animals (including humans).

TSWV is the type member of the genus *Tospovirus*, and is one of the most complex and studied plant pathogens, due to its peculiar biological and molecular characteristics that ensure a high capacity of adaptation to new hosts. The TSWV high genetic variability ensures its rapid and frequent evolution that enables the virus to overcome plant resistance. The viral particles are spherical or pleomorphic, with a diameter of 80-120 nm. Each viral particle is enveloped by a double-layer lipoprotein membrane that measures 5 nm in thickness. The membrane presents numerous pyriform protrusions of 5-10 nm, which are composed of glycoproteins, emerging from the surface. This envelope derives from the membranes of the Golgi apparatus or, occasionally, from the cytoplasmic membrane of the host cells. The glycoproteins are involved in the interaction between the virus and the insect. This structural conformation differentiates TSWV from all the other plant viruses and makes it more similar to the animal/human-infecting viruses.

TSWV presents a multipartite genome, composed of three ss-RNA molecules, respectively called: segment L (large) of about 8.900 bp, which encodes in negative sense the RNA-dependent RNA polymerase (RdRp); segment M (medium) of approximately 4.800 bp, which encodes in negative orientation the precursors of G1 and G2 glycoproteins, and in positive sense the non-structural protein NSm (+); segment S (Small) of about 3.000 bp, which encodes in positive sense the non-structural protein NSs (+), and in negative sense the N- protein that constitutes the envelope [3].

Like the TSWV, also the SARS-CoV-2 has a single-stranded RNA genome [4], of approximately 29,881 bp, encoding 9860 amino acids [5]. Gene fragments encode structural and nonstructural proteins. The S, E, M, and N genes encode structural proteins, whereas nonstructural proteins, such as 3-chymotrypsinlike protease, papain-like protease, and RNA-dependent RNA polymerase, are encoded by the open reading frame (ORF) region [6]. A large number of glycosylated S proteins cover the surface of SARS-CoV-2, these proteins are fundamental to the viral replication. The SARS-CoV-2 S proteins are very similar to TSWV G1 and G2

glycoproteins, and are highly conserved and are involved in receptor recognition, viral attachment, and entry into host cells [7].

2. MATERIALS & METHODS

2.1 Source of viral material

To verify the UV light effects on the inactivation of *Tomato spotted wilt virus*-viral particles, two UV lamps with different wavelengths were used, specifically, 222 nm and 254 nm. The TSWV-MDR/18 isolate was mechanically inoculated into three pepper plants (*Capsicum annuum* L.), to obtain a sufficient amount of TSWV-infected plant material to carry out the different assays. Inoculated plants were grown on a sterilized soil in an insect-proof glasshouse, with a photoperiod of 14 h of light and a target air temperature set at 28/20°C day/night. In detail, about 200 mg of fresh leaf tissue from an infected plant with MDR/18 isolate has been ground in a mortar with 6 ml of phosphate buffer pH 7 (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4 x $7H_2O$); the homogenate was distributed by rubbing on the plants leaf surface previously sprinkled with *Carborundum* (320 mesh), in order to cause micro-lesions and facilitate the passage of virions. The presence of TSWV was evaluated in all 3 plants by one step Retrotranscribed - quantitative Polymerase Chain Reaction (RT-qPCR) [8], after 30 days post inoculation (dpi).

2.2 Mechanical inoculation

The two UV lamps have been tested on TSWV sap extract obtained from the fresh leaf tissue of pepper plants resulted positive by RT-qPCR (Reverse Transcription-quantitative Polymerase Chain Reaction) for three different exposure times: 120, 150 and 180 seconds. A total of 6 different treatments were carried out. For each treatment, 3 pepper plants were mechanically inoculated, following the protocol described above. One healthy not-inoculated plant (MOCK) was used for each treatment as negative control. Moreover, one plant for each treatment was inoculated with TSWV sap extract not treated with UV light, used as positive control (POS). Inoculated plants were grown on a sterilized soil in an insect-proof glasshouse, with a photoperiod of 14 hours of light and a target air temperature set at 28/20°C day/night. To avoid the accidental virus spread, severe phytosanitary measures were applied, such as insect control and isolation. Symptoms of viral infection were recorded every 7 days, and the presence of TSWV was evaluated in all plants by serological and molecular analyses at 30 dpi.

2.3 Serological analysis

To verify the presence of TSWV on all inoculated, positive controls and healthy plants, two leaf tissues/plant were tested by Double-Antibody Sandwich Enzyme-Linked ImmunoSorbent Assay (DAS-ELISA), using specific monoclonal antibodies for TSWV (Agdia, Inc., Elkhart, IN), as described by Clark and Adams [9]. Results were obtained by measuring optical density (O.D.) values with a spectrophotometer at 405 nm wavelength, at one hour after the addition of the alkaline-phosphatase substrate solution to 96-well microplate.

2.4 Molecular analysis

In order to carry out the molecular analyses, total RNA was extracted from all inoculated, positive controls and healthy plants, using the Plant RNA/DNA Purification Kit (Norgen Biotek Corp., Ontario - Canada), following the manufacturer's instruction. Total RNA extracts were re-suspended in 30 µl of RNase-free water, and the RNA concentration was measured twice with NanoDrop ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilminutesgton, DE - USA); subsequently the RNA concentration was adjusted to approximately 10 ng/µl. The RT-qPCR assay was performed following the protocol described by Roberts and coworkers [8] in a Rotor-Gene Q2plex HRM Platform Thermal Cycler (Qiagen, Hilden, Germany). The reaction mix of 25 µl final volume contained one µl of total RNA extract with the concentration of ~10 ng RNA/µl, 200 nM forward (5'-GCTTGTTGAGGAAACTGGGAATT-3') and reverse primers (5'-GATGATGCAAAGTCTGTGAGGCT-3'), 100 nM TaqMan probe (5'-AAATCTAAGATTGCTTCCCACCCTTTGATTCAA-3'), 0.5 ml of RNase Inhibitor (Applied Biosystems, Foster City, CA, USA), 12 µl of 2X QuantiNova Probe RT-PCR Master Mix, 0.5 µl of QN Probe RT-Mix and H_2O DEPC water to reach final volume. Thermal cycling conditions were 45°C for 10 minutes for reverse transcription, 95°C for 10 minutes for RT inactivation, and 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The threshold has been set automatically by the thermal cycler software; the samples will be considered positive when they assume a Ct value ranging between 10.00 and 40.00. The Ct value corresponds at the cycle in which a significant increase in fluorescence occurs.

3. RESULTS AND DISCUSSION

3.1 Source of viral material

The three pepper plants inoculated with the MDR/18 isolate, used in order to obtain a sufficient amount of fresh tissue leaves necessary for the subsequent assays, gave positive results to RT-qPCR, performed at 30 dpi, with the following Ct values: 12.73, 14.51 and 15.97, respectively.

3.2 Mechanical inoculation

As regards mechanical inoculation of the MDR/18 isolate in pepper plants, none of them showed symptoms up to 30 dpi, except for one out of three plants, inoculated with sap extract treated with 254 nm UV light for 150 seconds, which began to show TSWV early symptoms at 15 dpi. Moreover, all inoculated plants used as positive controls showed the typical symptoms of TSWV infection at 13 dpi. The symptoms consisted mainly in yellowing of upper leaf surface with small necrotic spots, and speckled appearance with concentric chlorotic rings.

3.3 Serological analysis

The DAS-ELISA performed with specific monoclonal antibodies for TSWV showed that all inoculated and healthy plants were negative except for the only plant that showed symptoms described above. In detail, the spectrophotometer gave a O.D. values of 2.913 and 2.601, for the symptomatic and positive control plant, respectively. The O.D. values of

inoculated plants with untreated TSWV sap extract ranged from 2.789 to 3.102.

3.4 Molecular analysis

All inoculated plants tested at 30 dpi using RT-qPCR, following the protocol described above, were positive, regardless of the symptoms presence/absence and the type of UV treatments. Obtained Ct values ranged from 12.73 to 38.97 (Table 1). No amplification was obtained for MOCK plants, used as negative controls. As reported in Table 1 the lowest Ct value was obtained from the only symptomatic pepper plant. All plants inoculated with untreated TSWV sap extract resulted positive, with a Ct values ranged from 11.49 to 13.49.

Table 1. DAS-ELISA and RT-qPCR analyses results using different UV sap extract treatments

Id sample	UV wavelength (nm)	Exposure time (seconds)	Symptoms	DAS-ELISA (OD value)	RT-qPCR (Ct Value)
1	222	120	as	0.000	33.99
2			as	0.000	32.84
3			as	0.000	33.15
4		150	as	0.000	35.01
5			as	0.000	35.26
6			as	0.000	34.77
7		180	as	0.000	36.22
8			as	0.000	35.89
9			as	0.000	36.34
10	254	120	as	0.000	32.97
11			as	0.000	33.15
12			as	0.000	32.24
13		150	as	0.000	30.53
14			yls; ns; cr	2.913	12.73
15			as	0.000	31.22
16		180	as	0.000	31.84
17			as	0.000	32.14
18			as	0.000	32.02
MOCK/1	222	120	as	0.000	NTC
MOCK/2		150	as	0.000	NTC
MOCK/3		180	as	0.000	NTC
MOCK/4	254	120	as	0.000	NTC
MOCK/5		150	as	0.000	NTC
MOCK/6		180	as	0.000	NTC
POS/1	222	120	yls; ns; cr	2.987	13.93
POS/2		150	yls; ns; cr	3.082	12.07
POS/3		180	yls; ns; cr	3.011	12.56
POS/4	254	120	yls; ns; cr	3.102	11.98
POS/5		150	yls; ns; cr	2.954	13.23
POS/6		180	yls; ns; cr	2.789	11.49
Positive control (sap extract)	-	-	-	2.601	N.A.
Positive control (RNA)	-	-	-	N.A.	11.93

Notes: as: asymptomatic; yls: yellowing of upper leaf surface; ns: necrotic spots; cr: chlorotic rings; MOCK: negative control; POS: positive control inoculated with untreated TSWV sap extract; N.A.: not analyzed.

Results obtained show that all plants used as positive controls expressed the typical symptoms of TSWV infection, and were positive in both serological and molecular analyses. Plants inoculated with sap extract treated with different UV light, regardless of the wavelength and time of exposure, have not shown symptoms and were negative by DAS-ELISA assay, except for one plant (254 nm - 150 sec), which showed typical symptoms at 15 dpi and an O.D. value of 2.913. As regards the molecular assay, all treated plants were positive, even if with high Ct values compared to positive controls, except for the only symptomatic treated plant, that gave a Ct value of 12.73. These results suggest, presumably, that UV light treatment, regardless of wavelength and exposure time, influences the viral replication rate, reducing it significantly, in fact these plants showed no symptoms at 30 dpi. In the end, results showed that Lorenzini's hypothesis to treat Covid-19 patients comes out much stronger from our tests, who showed that indeed an exposition of a few tens of seconds of the TSWV-infected substance to UV-C rays reduces nearly to 0 the viral effect.

4. CONCLUSION

The results obtained in this work suggest that a similar treatment applied to SARS-CoV-2 can have the same or similar effect, with a good possibility of saving the lives to seriously ill patients. We can conclude that Lorenzini's hypothesis against Covid-19 is well posed and, if adopted, could save many lives: this becomes very important especially in this historical period, in which the developed countries will soon have the availability of a vaccine but, the poor countries probably not or not so soon and, so they could take big advantage of the therapy proposed, to save as many lives as possible.

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NOMENCLATURE

UV-C	Ultraviolet rays-C
TSWV	Tomato spotted wilt virus
RNA	Ribonucleic acid
ssRNA	Single-stranded RNA
nm	nanometer
RdRp	RNA-dependent RNA polymerase
bp	Bases pair
ORF	Open Reading Frame
RT-qPCR	Retrotranscribed - quantitative Polymerase Chain Reaction
mg	milligrams
dpi	days post inoculation
MOCK	healthy not-inoculated plant
POS	positive control
DAS-ELISA	Double-Antibody Sandwich Enzyme-Linked ImmunoSorbent Assay
O.D.	optical density
µl	microliter
ng	nanograms
nM	nanoMoles
H_2O	water
DEPC	diethylpyrocarbonate
Ct	Cycle threshold