Effects of modulation of potassium channels in Tobacco mosaic virus elimination

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Abstract

To evaluate the effects on modulation of K^+ channel in antiviral treatments, we report the application of potassium channel blockers (PCBs) tetraethylammonium chloride (TEA) and cesium chloride (Cs) in *in vitro* plantlets or callus cultures infected by Tobacco mosaic virus (TMV). The effect of PCBs were evaluated in: a) *in vitro* TMV-infection trials, where PCB-treatments were applied during infection process of healthy plantlet; b) chemotherapy trials, where PCB-treatments were applied in combination to antiviral drugs on TMV-infected plantlets; c) in antiviral trials using callus culture and sieving technique. Both PCBs were able to reduce relative concentration of virus [calculated as Fold Changes (FC) relative to control samples] during infection process, with major effect observed at 7 dpi and in medium or upper portions of plantlet. Positive effects of PCBs were

also observed following chemotherapy trials, where TEA significantly improves antiviral effectiveness of the antiviral drugs, causing higher FC compared to that obtained with just chemotherapy treatment since 3th subculture. Nevertheless, plantlets were positive to TMV up to 6th subculture, thus virus elimination was not achieved). Finally, the callus culture and sieving technique lead to 11.1±3.8 % of TMV-negative plantlets, while TEA-treated mother-calli lead to 22.2±3.8 % of negative plantlets. Concluding, findings suggest that modulation of K^+, previously indicated as essential in early stages of viral infection, could be also involved in antiviral techniques.

1. Introduction

In animal cells, ion channels are emerging as key factors required during virus replicative cycles, and have been assigned critical roles in virus entry, survival, and release (Hsu et al., 2004; Igloi et al., 2015; Sakurai et al., 2015). The selective transport of K^+ is involved in many physiological functions, including homeostasis of the membrane potential and the repolarization of the action potential in excitable cells (Hille, 2001). Studies in *Chlorella* lead also to suggest that a viral-encoded K^+ protein might be the evolutionary ancestor of all K^+ channel proteins (Thiel et al., 2013). The importance of K^+ channel activity during virus lifecycle was confirmed in virus families such as *Bunyaviridae* (Hover et al., 2016) and its role was also investigated in plants. In protoplast of *Gomphrena globosa* artificially infected by Papaya mosaic virus and Tobacco mosaic virus changes in average ion currents were observed, identifying an early event in the signal transduction pathway related to virus/host interaction (Schvarzstein, 1997). The central role of K^+ channel activity during early stage of infection was observed by Shabala et al. (2010). Excised mesophyll segments with removed epidermis were placed peeled side down directly on drop of purified Potato virus X suspension and changes in K^+ fluxes occurred within minutes from viral incubation. Unfortunately, the use of potassium channel blocker (PCB) to the leaf alongside virus did not

ameliorate infection symptoms after artificial foliar inoculation (Shabala et al., 2010) but no data about effect of PCB on virus movement and replication in tissues next to the infection sites was reported. The phenomenon of rapid K^+ release from host cells during the early phase of viral infection was also reported in infection of *Chlorella* cells by Paramecium bursari chlorella virus (Neupärtl et al., 2008).

In order to evaluate the modulation of K^+ channels in antiviral treatments, we report the effects of PCBs such as tetraethylammonium chloride (TEA) or cesium chloride (Cs) in *in vitro* systemic infection of Tobacco mosaic virus (TMV), one of most studied viruses which pathosystem still serves, as reported by Hammerschmidt (2015), as an excellent model for dissecting various aspects of plant–virus interactions. TEA (Volkov et al., 2005; Volkov, 2006) and Cs (White and Broadley, 2000; Zhu and Smolders, 2000) are blockers of ionic channels and their application decreased plant K^+ uptake compared with the control treatment (Pacheco-Arjona et al., 2011). The effect of PCBs were evaluated in: a) *in vitro* TMV-infection trials, where PCB-treatments were applied during infection process of healthy plantlet; b) chemotherapy trials, where PCB-treatments were applied in combination to antiviral drugs on TMV-infected plantlets; c) in antiviral trials using callus culture and sieving technique.

2. Materials and Methods

2.1. TMV production, purification and assay

A collection of virus isolates of TMV was maintained in tobacco plants (*Nicotiana tabacum* cv. Turkish) under greenhouse conditions (18-24 °C), insect free. The plants, infected by an isolate of virus type-strain identified by RT-PCR (Luvisi et al., 2015a), were used as the inoculum source of viruses. Viral particles were purified from diseased *N. tabacum* plants form leaves (50g of tissue) (Uhde-Holzem *et al*., 2010). According to Rettcher *et al.* (2015), polyethylene glycol (PEG)

precipitation method was performed as reported by Uhde-Holzem *et al*. (2010) omitting the sucrose cushion centrifugation to limit losses in TMV amount (Luvisi et al., 2017). Following the sucrose gradient step, selected fractions were pooled, followed by centrifugation at 248,000 × g for three hours. The TMV amount in the final sample was assessed by reading at wavelength of 260 nm (OD_{260}) applying the viral extinction coefficient of 3.0. TMV quantity in plantlets or callus culture was esteemed by qRT-PCR employing a CFX96 Real-Time thermocycler (Bio-rad, USA), following Edelbaum *et al*. (2009). TMV primers designed by Edelbaum *et al*. (2009) were used to amplify 173 bp segment. Quantitative PCR assays were carried out on 70 ng of tobacco total RNA. The PCR was set in order to achieve 15 min activation at 95 °C, 45 cycles of 20 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C and 20 s at 82 °C (melting: ramp from 60 to 99 °C with 1°C of temperature increase every 5 s). Threshold cycle value (Ct) was set at 35 to discriminate between healthy or TMV-infected samples. Prior to use purified TMV as inoculum for *in vitro* plantlets or callus culture, the virus was diluted in sterile 0.01 M sodium phosphate buffer.

2.2. In vitro *plantlets*

In vitro tobacco plantlets were obtained following Panattoni et al. (2015). Nodes from healthy or TMV-infected plants were collected and surface sterilized before transfer to aseptic culture tubes with Murashige and Skoog (MS) (1962) growth medium. Plantlets were maintained in a controlled environment chamber that assured maintenance of health condition, with a temperature regime of 22±1 °C, 16 h photoperiod, 50 $\mu Em^{-2}\ s^{-1}$; plantlets were transferred to fresh growth medium at 14-day intervals. After an acclimatization period of two months, the health condition of each plantlet was confirmed by qRT-PCR. Plantlets with three nodes were used to evaluate the effect of PCBs in *in vitro* TMV-infection trials, where PCB-treatments were applied during infection process of healthy plantlet and in chemotherapy trials, where PCB-treatments were applied in combination to antiviral drugs on TMV-infected plantlets.

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106 *2.3. Effect of PCB in* in vitro *TMV-infection trials*

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108 Effects of PCBs in *in vitro* TMV-infection trials were evaluated using the following growth media:
109 TMV medium (MS medium with 4.5 µg mL^{-1} of TMV); TMV-TEA 20/40 (MS medium with 4.5
110 µg mL^{-1} of TMV and 20 or 40 mM of TEA); TMV-Cs 10/20 (MS medium with 4.5 µg mL^{-1} of
111 TMV and 10 or 20 mM of Cs); TEA 20/40 (MS medium with 20 or 40 mM of TEA); Cs 10/20 (MS
112 medium with 10 or 20 mM of Cs); MS medium. Healthy explants were cultivated in a 2-step
113 process. In the first step (inoculation), the plantlet were transferred on TMV-PCB (Fig. 1A) or
114 TMV-medium (Fig. 1B), and cultured for 24 hours. After infection treatment (second step of *in*
115 *vitro* culture), plantlets were transferred to PCB medium or MS medium for 14 days of growth. All
116 experiments were performed in triplicate; each experiment consisted of 15 *in vitro* plantlets.

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118 *2.4. Effect of PCB in chemotherapy trials*

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120 For chemotherapy trials, *in vitro* tobacco explants were obtained from artificially TMV-infected
121 *Nicotiana tabacum* L. cv. Turkish. Antiviral chemicals such as ribavirin (RB) (Panattoni et al.,
122 2013a; 2013b) and mycophenolic acid (MPA) (Rinaldelli et al., 2012; Guazzelli et al., 2015; Luvisi
123 et al., 2015b) (Sigma–Aldrich, St. Louis, MO) were tested individually against TMV in TEA-
124 addicted (20 mM) growth medium (Fig. 1C). Growth media with antiviral chemicals without TEA
125 or with TEA only were used as control (Fig. 1C). Drugs were hydrated in stock solution and,
126 immediately before use, ultra-filtered and added to proliferation medium after sterilization (0.10
127 mM for RB and 0.40 mM for MPA) (Luvisi et al., 2012). The experimental design involved growth
128 of infected plantlets for six consecutive subcultures (each one 14-days long). After each subculture,
129 the apical portion (1 cm) of each plantlet was transferred to fresh growth medium and the residue

was assayed by qRT-PCR (Luvisi et al., 2011). All experiments were performed in triplicate; each experiment consisted of 15 *in vitro* plantlets.

2.5. Effect of PCB in callus culture and sieving technique

Following Kwon et al. (2012), callus cultures were initiated from virus-infected leaves and grown on control medium containing MS salts, Gamborg's B5 vitamins (Gamborg et al., 1968), 30 g/l sucrose, 1 mg/l benzylaminopurine, 0.1 mg/l naphthaleneacetic acid, and 8 g/l phytoagar. The treatment medium differs from control medium by adding TEA (20-40 mM). Calli were grown for 8 weeks in a growth chamber under the conditions described above for plant growth until they reached a size >1 cm. TMV-infected callus (mother callus) was divided in small callus particles (micro-calli) using the Cell Dissociation Sieve (Sigma-Aldrich, Inc., St. Louis, MO) under sterile conditions (Fig. 1D). As reported by Kwon et al. (2012) the micro-calli (250 μm in diameter) were transferred for callus proliferation to semisolid media (3 g/l phytoagar). After 6 weeks, both control calli than TEA-treated calli were transferred solid MS media for further growth. Shoot induction occurred within 3-4 weeks of culture and health status was checked. All experiments were performed in triplicate; each experiment consisted of 15 micro-calli.

2.6. Virus assay

Sampling was carried out during the following 7 and 14 days after transplanting, considering tissues (stem and leaves) of the lower, medium or upper node. For the lower node analysis, portion of stem tissues in contact with medium was removed prior analysis. TMV extraction and quantification was achieved following Edelbaum et al. (2009). Virus amounts were determined by qRT-PCR using a CFX96 Real-Time thermocycler (Bio-rad, USA), coupled with the DNA-minor-groove binding fluorescent dye SYBR Green I. Relative quantification was calculated using the comparative cycle

156 threshold (Ct) method in which the change in the amount of the target viral RNA was normalized in
157 relation to the level of infection of control plantlets, chosen as a baseline. Thus, the relative level of
158 infection of the treated plantlet was calculated as Fold Changes (FC) relative to control samples.
159 Specific primers by Edelbaum et al. (2009) were used to amplify 173 bp segment. qRT-PCR was
160 performed using 70 ng of plant total RNA. The cycling conditions were 15 min activation at 95 °C,
161 45 cycles of 20 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C and 20 s at 82 °C (melting: ramp from 60 to
162 99 °C, rising by 1 °C each step, waiting 5 s for each step thereafter).
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164 *2.7. Statistical analysis*
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166 Data were elaborated using Sigma-Plot software (version 11; Systat Software, San Jose, CA). The
167 software was used to perform analysis of variance (ANOVA). To evaluate differences in virus
168 concentration, T-test was used to compare fold changes between treated or untreated plantlets. Data
169 expressed in percent were converted in arcsin values. $P \leq 0.05$ was considered to be significant.
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171 **3. Results**
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173 *3.1. Effect of PCB in TMV-infection trials*
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175 Treatment with TEA or Cs did not prevent plantlet infection by TMV. The virus was found in each
176 partition of plantlet since 7 days post inoculation (dpi), thus systemic distribution of virus was
177 achieved. However, both PCBs were able to reduce relative concentration of virus, with major
178 effect observed at 7 dpi and in medium or upper portions of plantlet. FC_{medium} and FC_{upper} was set at
179 1.2 or 1.4 at 7 dpi by Cs 10 or Cs 20 treatment, respectively (Fig. 2A, B). Nevertheless, relative
180 concentration of TMV come closer to untreated plants at 14 dpi, when FC was set at 1.0-1.1 (Fig.
181 2C, D). A similar TMV distribution was observed when infection was carried out with TEA

182 treatment. FC_{medium} and FC_{upper} was set at 1.3 at 7 dpi by TEA 20 treatment (Fig. 2C), while 1.5 was
183 achieved with TEA 40 medium (Fig. 2D). Similarly to CS tests, relative concentration of virus at 14
184 dpi was comparable to control, as point out by FC about 1.0-1.2 (Fig. 2C, D).
185 Even if the higher concentration for both PCB (Cs 20 and TEA 40) lead to higher FC – and
186 presumably lower virus concentration - , phytotoxic effects were observed, because plantlets did not
187 growth and chlorosis were observed in more than half of plantlets at 14 dpi. Conversely, at lower
188 concentration (Cs 10, TEA 20), chlorosis involved less than 15 % of plantlets.
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190 *3.2. Effect of PCB in chemotherapy tests*
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192 FC_{RB} was set at 1.2 at 4th subculture, suggesting a reduction of TMV caused by antiviral drug (Fig.
193 3A). TEA significantly improves antiviral effectiveness of RB, causing higher FC_{TEA} than FC_{RB}
194 since 3th subculture. Nevertheless, plantlets were positive to TMV up to 6th subculture, thus virus
195 elimination was not achieved. A similar behavior of TEA was observed in MPA-treated plantlets.
196 The antiviral drug slightly reduce relative quantity of TMV ($FC_{MPA} \approx 1.1$ from 1st to 5th subculture,
197 1.2 at 6th subculture), but significant effects were observed in mixed treatment with TEA since 3th
198 subculture (Fig. 3B). However, TMV was not eliminated from plantlets. Relative concentration of
199 TMV was not altered by TEA-addicted medium without antiviral drugs (data not shown).
200 In RB-treated plantlets (w/o PCB), chlorosis were observed in more than half of plantlets since 4th
201 subculture, confirming toxic effect of RB. MPA-treated plantlets did not showed toxic effect during
202 whole trial, while chlorosis were observed in most of MPA/PCB treated plants (73 %).
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204 *3.3. Effect of PCB in callus culture and sieving technique*
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206 About 80% of micro–calli obtained by TEA 20-treated mother callus produced shoots within a
207 month from transfer, compared to 95% of micro-calli obtained from untreated mother callus (Tab.

208 1). Conversely, production of micro-calli obtained from TEA 40-treated mother callus was very low
209 compared to untreated ones, with shoots induced in about 22% of survived calli after transfer on
210 solid media (Tab. 1). The callus culture and sieving technique lead to 11.1±3.8 % of TMV-negative
211 plantlets, while TEA 20-treated mother-calli lead to 22.2±3.8% of negative plantlets. Virus-free
212 plantlets out of total treated mother-calli was very low using 40 mM of TEA, due to low rate of
213 callus survival.
214
215 **4. Discussion**
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217 In tobacco cells, TEA is a very efficient K^+ channel blocker (90% inhibition) followed by Cs (75%)
218 (Shabala et al., 2010). While *in vivo* foliar treatments associated to mechanical inoculation did not
219 ameliorate symptoms on tobacco leaves (Shabala et al., 2010), we observed reductions in relative
220 quantity of viruses in plantlets grown on infected medium added with PCBs. TMV-infection trials
221 showed that higher dosage of PCB slight increase FC in the whole plantlet at 7 dpi, and the higher
222 FC_{medium} and FC_{upper} compared to FC_{lower} suggest that PCBs interfere with virus. Milder differences
223 were also observed at 14 dpi, when TMV seems to replicate quite homogenously within plantlet.
224 These results suggest that the lower portions of plantlet were sensitive to TMV, probably due to
225 proximity to excised tissues partially submerged by infected growth medium. This wound
226 represents the first infection site and absorption of PCBs may results ineffective in preserving its
227 infection, probably because the blocking effect of pharmacological agents was only partial, as
228 suggested by Shabala et al. (2010). Conversely, in the upper parts of plantlet, the PCB adsorbed
229 since 1 dpi may slow down virus propagation among tissues, at least during first days after
230 inoculation. In our trials, plantlets exposure to TMV lasted 24 hours and it directly affected basal
231 tissues. Since day 2, plantlets were transferred on PCB-addicted medium that can be absorbed by
232 whole plant, while virus movement to upper tissues could be relayed mainly in long-distance
233 movement which cause new infection sites to disseminate the virus efficiently throughout the whole

234 plant, as reviewed by Hipper et al. (2013). Ion homeostasis of cells involved in new infection sites
235 could be altered by PCB continuously absorbed by plantlet, interfering with virus life cycle.
236 In chemotherapy tests here reported, where antiviral drugs showed partial effectiveness (Luvisi et
237 al., 2012), targeting K^+ seems lead to positive results, due to significant reduction in fold change
238 caused by TEA in RB/MPA-addicted medium. In animal-related *Bunyaviridae* virus, K^+ sensitive
239 step does not include virus binding/entry, but it is most likely that K^+ channel modulation
240 contributes to early events within the virus life cycle including virus uncoating or events prior to the
241 formation of RNA replication factories (Hover et al., 2016). Targeting these K^+ channels in patients
242 infected with virus infections may therefore represent a new prospect for future antiviral drug
243 development (Hover et al., 2016), but no data is available in order to suggest a similar behavior in
244 plants, where elimination of TMV from tobacco plants via chemotherapy is very difficult to
245 achieve.
246 With regards to effects of PCBs in callus culture, the increased efficiency of callus-mediated virus
247 elimination caused by TEA should, at least partially, be explained by strong alteration of K^+ flux,
248 that modulate cell turgor (Shabala et al., 2010). Callus culture was applied to eliminate viruses in
249 several plant species (Goussard and Wiid 1992; Niimi et al. 2001; Gambino et al. 2006; Sharma et
250 al. 2008) and the heterogeneous distribution of virus (Scagliusi et al. 2002) can play a significant
251 role in antiviral effectiveness. Thus, the uneven distribution of the virus in mother calli was
252 proposed to be due to the production of virus-infected and virus-free cell clusters during the callus
253 disruption process (Kwon et al., 2012), explaining the results of previous studies showing the
254 regeneration of virus-free plantlets from callus culture by multiple steps of subculture or production
255 of TMV virus-free tobacco plantlets only from smaller-sized callus cultures (Toyoda et al. 1985).
256 As viruses can translocate between cells at a slow pace via plasmodesmata, Kwon et al. (2012)
257 suggested that the invasion of viruses through plasmodesmata was limited to the older cells in the
258 callus, while the rapidly proliferating young cells escaped virus invasion. Thus, the effectiveness of
259 virus cell-to-cell movement via plasmodesmata (Hipper et al., 2013) may play a significant role in

virus elimination. Intercellular transport is modulated by cell turgor and passage can be blocked at high differential pressure between cells (Oparka and Prior, 1992).

Concluding, findings suggest that modulation of K^+, previously indicated as essential in early stages of viral infection, could be also involved in antiviral techniques.

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Fig. 1. Effect of PCBs in *in vitro* TMV-infection trials were evaluated using healthy tobacco plantlet cultured for 24 hours TMV-PCB medium and transferred on PCB medium for 14 days of growth (A). Control plantlet , after 24-hours of growth on TMV-medium, where transferred to MS medium for 14 days of growth (B). Effect of PCBs in chemotherapy test were evaluated after six consecutive subcultures (each one 14-days long) on PCB-antiviral drug medium, antiviral drug medium or PCB medium (C). Effect of PCB in callus culture dividing infected mother callus growth on PCB medium into micro-calli, followed by micro-calli growth on semi-solid medium and shoot induction (D).

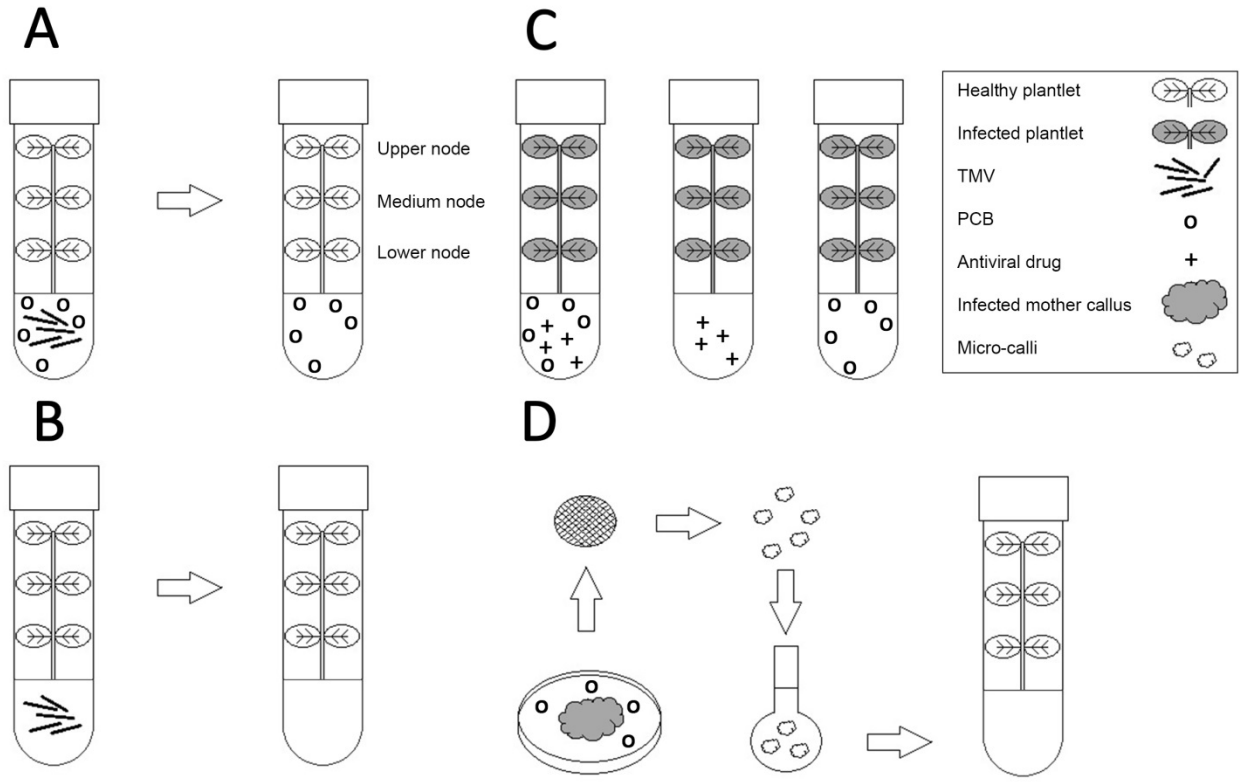


Fig. 2. Levels of Tobacco mosaic virus in lower, medium or upper node of *in vitro* tobacco plantlets expressed as relative fold change to control plantlets. Healthy plantlets were grown for 24 hours in TMV-infected medium added with $CsCl_2$ (Cs, 10-20 mM) (A, B) or tetraethylammonium chloride (TEA, 20-40 mM) (C, D) or without Cs/TEA addiction (control plantlets). Plantlets were transferred in growth medium for virus assay at 7 or 14 days post inoculum (dpi). The means ± SE were calculated from data pooled from 15 in vitro plantlets and experiments were performed in triplicate.

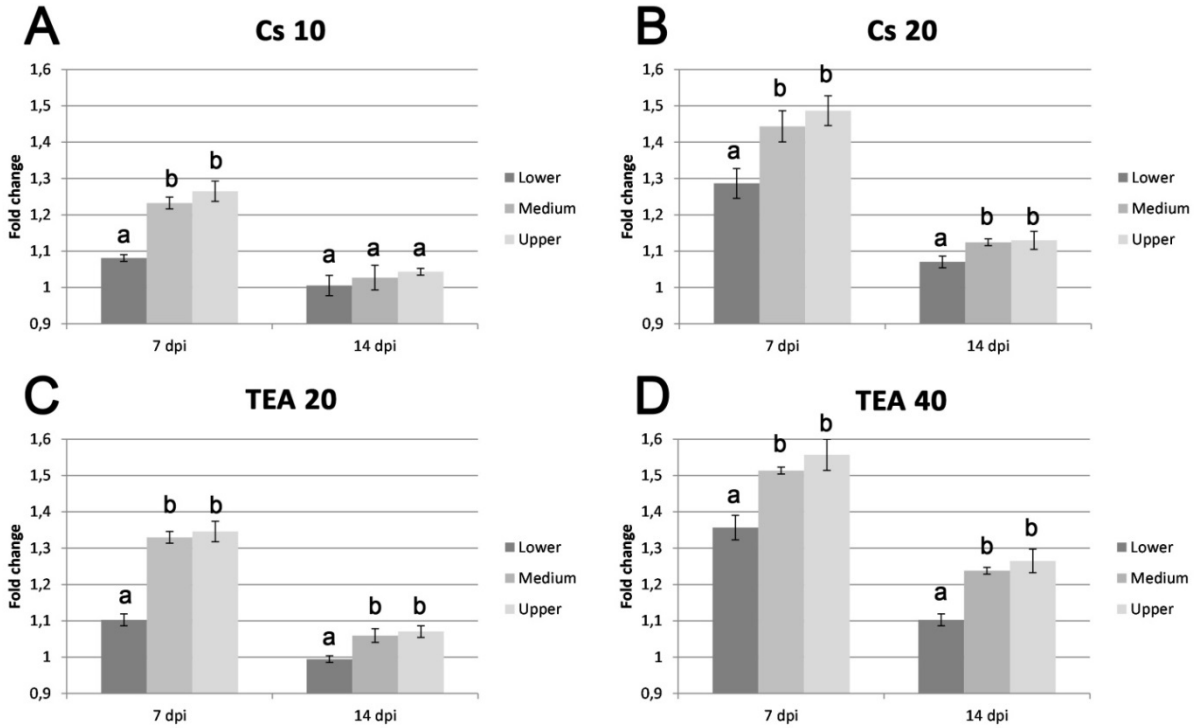
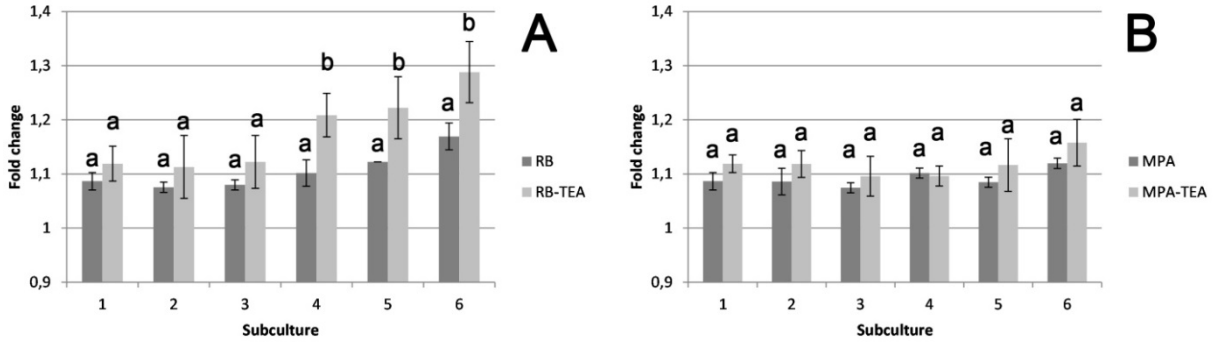


Fig. 3. Levels of Tobacco mosaic virus (TMV) in infected *in vitro* tobacco plantlets expressed as relative fold change to untreated TMV-infected plantlets after each 14-day length subculture in medium added with tetraethylammonium chloride (TEA, 20 mM) alone or with the antiviral chemicals RB and MPA. The means ± SE were calculated from data pooled from 15 *in vitro* plantlets and experiments were performed in triplicate.



Tab. 1. Plantlet production and TMV-negative (restored) plantlet derived from TMV-infected mother-callus cultured on growth medium added with tetraethylammonium chloride (TEA, 20-40 mM), compared to mother-callus growth on untreated medium (control). The means ± SE were calculated from data pooled from 15 micro-calli and experiments were performed in triplicate.

	No. plantlets		**% of restored plantlets**	
	Survivors	Restored	Out of survivors	Out of total
Control	14.3±1.2 (15) a	1.7±0.6 b	11.5±3.2 b	11.1±3.8 b
TEA 20	12.0±1.0 (15) a	3.3±0.6 a	27.8±5.2 a	22.2±3.8 a
TEA 40	2.7±0.6 (15) b	0.7±0.6 c	22.2±19.2 ab	4.4±3.8 c