Evaluation of Seed Re-treatment Efficiency for Viroid Positive Seed Lots

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Abstract

Viroid is a small single stranded circular RNA molecule without protein coat. Because of no coding capacity, it needs to use the RNA polymerase of hosts to replicate itself. It can be disseminated by mechanical transfer, insects, pollen, seeds, etc. International seeds trading are usually long distance, so easily for viroid to be disseminated from countries to countries. Viroids make several economically significant diseases but sometimes it will not induce symptoms, so many countries set quarantine requirements and test imported seeds to avoid viroid spreading. Pospiviroidae, one family of viroid, usually infects Solanaceae crops, e.g. tomato, pepper, potato. In last ten years, there are some disinfectants mentioned in previous study effective for disinfecting seeds to diminish viroid. In this study, three tomato lines and two pepper lines were selected because they were detected viroid-infected after the first disinfection. To do seeds re-treatment, using different concentration of NaOCl and combination of HCl /Trisodium phosphate (TSP) were used to treat seeds with different concentration and time to make totally eight treatments. Infection rate of each line at different treatments were checked by reverse-transcription polymerase chain reaction (RT-PCR) after seeds secondary disinfection. After seeds disinfection, infection rate and germination rate were inspected and analyzed. Infection rate was based on the ratio of infection. Germination rate was determined by when did the cotyledon of seed grow and evaluated whether using those disinfection would destroy seed vigor. According to our data, in tomato lines, using low concentration of NaOCl to disinfect seeds could get lower infection rate instead of using high concentration one, and also have a good germination rate. In pepper lines, using combination of HCl/TSP to disinfect seeds could get better disinfection efficiency, in addition, the germination remain well. In this study, we found the trend that using which treatment is moderate for tomato and pepper, but the best concentration disinfectant and treatment duration combination could be study further in the future.

Keywords: Viroid, Re-treatment, disinfectant

1. Introduction

Viroids are the smallest plant pathogen known currently, consisting of naked, single-stranded, closed circular RNAs, with a high degree of secondary structure, which do not code for any known proteins or peptides [1]. Viroid could be divided into Pospiviroidae and Avsunviroidae. The genus Pospiviroid, one of the five genera within the family Pospiviroidae, includes nine species, of which *Potato spindle tuber viroid* (PSTVd) is the type member [2, 3]. All pospiviroid species, except iresine viroid 1, are able to infect at least one of the main solanaceous crops, e.g. pepper, potato, and tomato [4]. Pospiviroid usually induces serious economically disease, for instance, making plants stunting, many foliage symptoms, and even symptomless so that it is hard to notice sources of infection [5]. Pospiviroids can be spread by vegetative propagation and transmission via mechanical transfer (contact), insects, pollen, and seeds [6, 7], especially, several countries consider seeds as an important pathway for the introduction of pospiviroids in commercial pepper and tomato crops. Because production and processing occur at a global scale, this has led to the stringent seed-testing and quarantine requirements for seed lots international movement [8].

In recent years, several studies conducted to identify disinfectants that reduce the infectivity of plant viruses and viroids have been reported for ornamental plants [9, 10, 11]. Several common disinfectants (e.g. NaOCl and nonfat dry milk) have been shown to be effective in preventing the spread. NaOCl is useful for diminishing the PSTVd. However, currently there is still no clear understanding as to the effectiveness of disinfectants against a range of viruses and viroids encountered in greenhouse tomato productions [12]. Reverse-transcription polymerase chain reaction (RT-PCR) is used routinely to detect PSTVd in potato tubers, leaves, pollen, and botanical seed [13]; find newly discovered PSTVd strains [14, 15]; and, experimentally, to detect PSTVd in environmental samples (e.g., swabs from concrete and glasshouse structures and plants)[16]. However, it cannot differentiate between infectious and inactivated viroids, particularly in environmental samples where RT-PCR could amplify viroids inactivated by disinfectants. Thus, wherever possible, effective infectivity tests should also be used in addition to RT-PCR.

In this study, the viroid-infected tomato and pepper seeds were re-treated by some disinfectants usually used to diminish viroid, and evaluated which combination is the best and confirmed by conducting RNA extraction and RT-PCR. In addition, the germination rate would were tested to ensure the disinfectants did not destroy the vigor of seeds after the disinfection.

2. Materials and Methods

2.1. Seeds preparation

All seeds were obtained from Genetic Resources and Seed Unit (GRSU) in the World Vegetable Center (WorldVeg). In total, there are five lines, three are *Solanum lycopersicum* (tomato), and the code are AVTO1818, VI005930, VI005930 respectively; two are *Capsicum annuum* L. (pepper), the code are VI050599 and AVPP1324. Those seeds were chosen because they were detected viroid-positive and the quantity of seeds was enough for experiment. All seeds were disinfected by 0.5N HCl and 10% trisodium phosphate (TSP) for 15 minutes and 1 hour respectively in the first disinfection, but still detected viroid-positive. There were eight treatments conducted per line (Table1), and five repetitions in each treatment. 450

seeds of each sample for *Solanum lycopersicum* and 250 seeds of each sample for *Capsicum annuum* L. were prepared then put in mesh bags to do disinfection. For saving the time, just first subsample of each line was counted for the accurate number of seeds, the others were measured by weight following the first subsample.

2.2 Seeds disinfection

In Table 1, a-d treatment, seeds were washed by flowing water for 15 minutes and dried for 2 minutes after NaOCl treatment (CLOROX, USA). e-g treatment, seeds were washed by flowing water for 15 minutes and dried for about 2 minutes after HCl and TSP treatment. Seeds were only washed by flowing water for 75 minutes in h treatment for the mock control of totally eight treatments. When all steps were done, seeds were put into the 15℃ drying room for a week. (Fig. 1)

2.3 Germination rate

After seeds drying, 50 complete seeds of each subsamples were selected and sowed on filter paper which was in petri dishes then put into growth chamber and be observed. The environment of growth chamber was set in dark and 25℃. The germination condition was recorded at the seventh and the fourteenth day after sowing. When cotyledon expanded from seed coat, the seeds were considered to be germinated (Fig. 2). The germination rate was the ratio of budding seeds in 50 seeds at the fourteenth day.

2.4 Infection rate

2.4.1 RNA extraction

The rest seeds of each subsample were tested independently and were crushed to powder in a stainless steel tube with stainless steel beads by a homogenizer, then transferred to a plastic bottle and added buffer 1 [0.2 M Tris-HCl pH 8.0 , 1.0 M NaCl], 2-mercaptoethanol (β-ME), buffer 2 [0.1 M EDTA pH 8.0, 2.5 g/100 ml Sodium lauryl sulphate, 6.6 g/100 ml PVP-40], and extraction buffer [buffer 1 and buffer 2 mixed 1:1] in order. Especially, the ratio of buffer 1, buffer 2, and extraction buffer remain 1:1:10 according to the seeds weight, then shook gently and kept on ice until all subsamples are done. To do RNA extraction, added 1.5 ml supernatant of seed extraction to 2 ml centrifuge tube and heated at 65℃ for 10 minutes. Added 500 ul 5 M potassium acetate in tube then mixed homogeneously and kept on ice for 30 minutes. Followed by centrifugation at 10,000 rpm for 10 minutes at 0℃. The 900 ul aqueous phase was collected into 1.5 ml centrifuge tube with the addition of 540 ul isopropyl alcohol and made it well mixed then keep on ice for 30 minutes. Followed by centrifugation at 10,000 rpm for 10 minutes at 0℃ again, then used 700 ul 70% alcohol to wash the pellet, next, used the different volume of pipetmans to remove the liquid except pellets and centrifuged at 10,000 rpm for seconds at 0℃ after removing. The obtained pellets were air-dried aseptically and were dissolved in 50 ul of RNase-free water then test the quality of RNA by NanoDrop (Thermo Fisher Scientific Inc., USA).

2.4.2 RT-PCR

RT-PCR were conducted with two sets of primers (Posipi1F/1R [17] and Vd6up/Vd4dw2 [Unpublished, Authorized by Taiwan Agricultural Research Institute, TARI]) (Table 2). Line A, B, D, E were tested for Pospiviroids and line C was tested for CLVd. Another primer set (MT-F2/R1 [18]) (Table 2) was used for ensuring if RNA was from plant as internal control; it was run in all lines in parallel. Following is the program of RT-PCR for viroid. Added 4.3 ul mixture A [3.5 ul ddH_2O, 0.4 ul 10 mM Pospi1F, 0.4 ul 10 mM Pospi1R] into 200 ul PCR tube, and mixed with 1 ul RNA with filter tip individually. A mock control and a positive control should be added in each test, next, heated reagent with RNA to 95℃ for 5 minutes, and transfer directly onto ice to cool down immediately for 5 minutes. Then, the whole program adding 6.7 ul mixture B [6 ul 2X Kit mix, 0.7 ul 5X Band Doctor] into PCR tube should be performed on ice, and mixed well by pipetting gently. Finally, put tubes back into PCR machine and start the reverse transcription step, followed by 5 cycles of DNA denaturation at 95℃ for 20 s, annealing at 68℃ for 40 s and extension at 72℃ for 1 min. Next, followed by 35 cycles of DNA denaturation at 95℃ for 20 s, annealing at 63℃ for 40 s and extension at 72℃ for 1 min, finally, keep at 16℃ after the last cycle 72℃ for 5 min. After finishing RT-PCR program, PCR products were collected for gel electrophoresis. A 2% agarose gel were used for analysis. The kits were changed to 0.3 ul 10 mM Vd6up and 0.5 ul 10 mM Vd4dw2 if it was line C. About the program for internal control was mention below. First, mixing the solution [4.2 ul ddH_2O, 0.4 ul 10 mM MT-F2, 0.4 ul 10 mM MT-R1, 6 ul 2X Kit mix] and put into 200 ul PCR tube before adding 1ul RNA with filter tip individually. A mock control and a positive control should be added in each test and mixed well by pipetting gently, next, put into PCR machine and start the reverse transcription step, followed by 1 cycles at 55℃ for 30 min, then 95℃ for 2 minutes, next, followed by 35 cycles of DNA denaturation at 94℃ for 30 s, annealing at 58℃ for 30 s and extension at 72℃ for 1 min. finally, keep at 16℃ after the last cycle 72℃ for 5 min. After finishing RT-PCR program, PCR products were collected for gel electrophoresis. A 2% agarose gel were used for analysis.

2.5 Statistical analysis

Values were expressed as a decimal point which rounded to three decimal places, and measurements were obtained in five repetitions. The significant difference was determined at the $p < 0.05$ level for Tukey test by R software (version 4.1.0).

Table 1 Application rate of all disinfectants and the time seeds treated.

	Treatment
a	1% NaOCl for 10min
b	1% NaOCl for 30min
c	5% NaOCl for 10min
d	5% NaOCl for 30min
e	0.5 N HCl for 15min and 10% TSP for 1hr
f	0.5 N HCl for 30min and 10% TSP for 2hr
g	0.65 N HCl for 15min and 20% TSP for 1hr
h	running water (mock control)

Table 2 Primers used in the present study for the detection and amplifcation of Pospiviroids and PSTVd

Viroid name	Primer	Sequence	Amplicon size
Pospi viroids	Pospi 1 F	5’-GGG ATC CCC GGG GAA AC-3’	196-288 bp
	Pospi 1 R	5’-AGC TTC AGT TGT WTC CAC CGG GT-3’	
CLVd	Vd6up	Unpublished, Authorized by TARI	~200bp
	Vd4dw2	Unpublished, Authorized by TARI	
Internal control	MT-F2	5'-GCT TCT TGG GGC TTC TTG TTC GAT A-3'	185 bp
	MT-R1	5'-ATC TCC AGT CAC CAA CAT TGG CAT-3'	

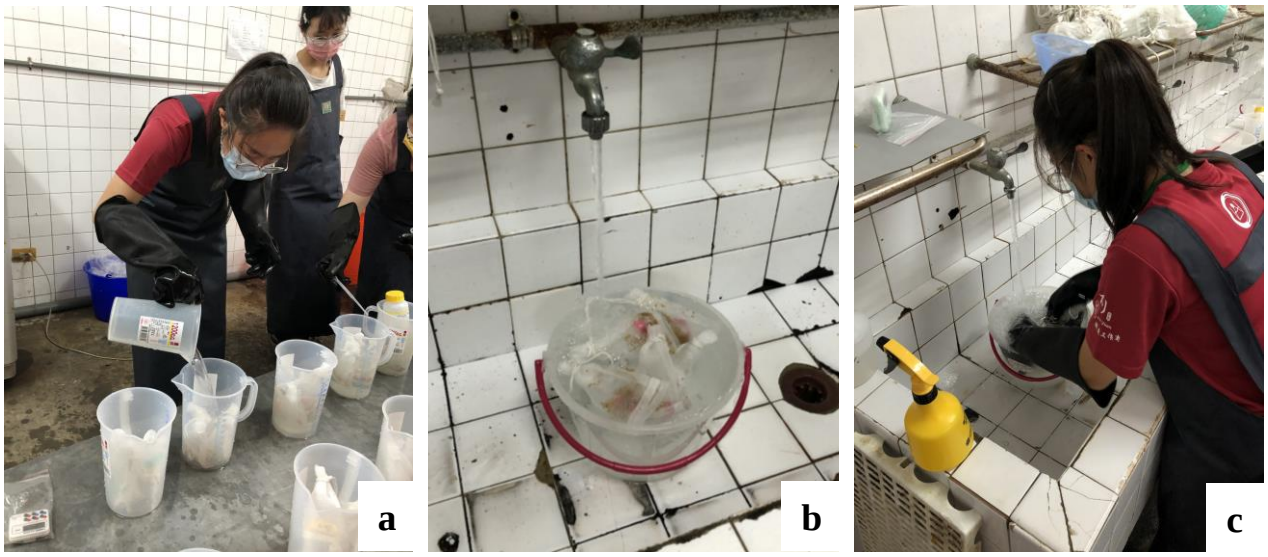

Fig. 1 (a) Soaking seeds in solution of disinfectant. (b), (c) using running water to wash treated seeds.

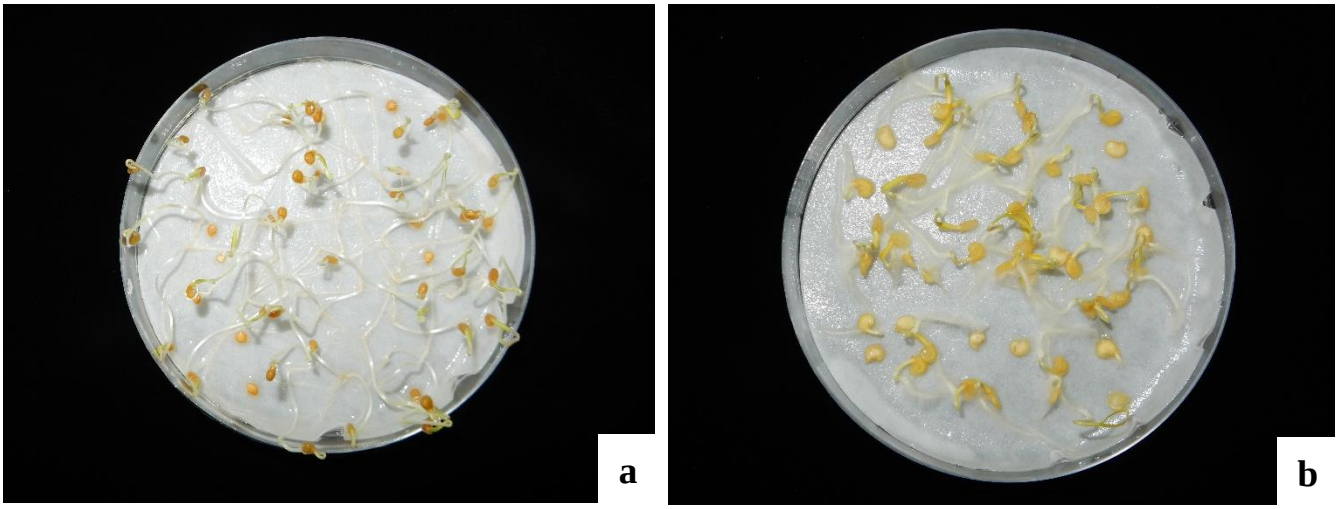

Fig. 2 Cotyledon expanded from seed coat at the seventh day. (a) tomato. (b) pepper.

3. Result

3.1 Infection rate

The degree of infection after disinfection are different. The viroid in line C were almost removed but there are some parts still infected in line E. Actually, mock controls were not all remain positive. It means that the degree of infection were not very uniform in each line. According to our data, the trend was found that each treatment is suitable for tomato lines to disinfect seeds except for c, d and e treatment. And it seems that using HCl and TSP could achieve disinfection effect and got the low infection rate in pepper lines. It seems that using lower concentration of NaOCl is suitable for line B to disinfect seeds instead of using higher concentration of NaOCl. e and f treatment in line B and line E, using same concentration but soaking seeds for a longer time could get the same effect. (Table 3)

3.2 Germination rate

In line A, the germination rate in g treatment is the worst, and in c treatment is the best. The data shown that there is no difference between treatment b, c and d. So using low concentration of NaOCl for longer time or higher concentration of NaOCl may not destroy seeds germination in line A. In line B, there were no difference in all treatments. In line C, seeds treated in high concentration of NaOCl and HCl with TSP for longer time shown low germination rate, conversely, it got higher germination rate in those low concentration treatments. In line D, there were no difference in treatments except for d treatment. It was considered that seeds may hurt by high concentration of NaOCl especially for a longer time. In line E, the germination rate in c and d treatments were not reasonable. The germination rate in d treatment are very low in line D and E, so it is speculated that high concentration of NaOCl may not be a suitable disinfectant for pepper. (Table 4) In addition, there are nearly no seeds of pepper in d treatment germinated at the seventh day. In line E, seeds treated with f treatment had the best germination compared with d treatment. There is an obvious difference (Fig. 3). There was the same situation in line D at the seventh day.

3.3 Statistical analysis

Charts of germination rates treated with different treatments were plotted (Fig. 4). The chart shows the mean and the range of standard deviation. In line D and line E, the germination rates in d treatment are not ideal at the seventh day. In line E, there is no difference at the fourteenth day, but in line D, there were two plates of seeds grew well. The wider standard deviation means the lager the value affected the whole data at same treatment. D-1 shown the situation in normal and D-2 shown data deleting 3 pieces of the data which were poorer than others in the same 5 repetitions. D-1 shown that because of the large standard deviation in d treatment, the mean of d treatment would become lower, then means of other treatments while they were showing no difference comparing to each other. The chart of D-2 shown more normal than D-1, and there was significant difference between treatments (Table 5). To remain the completion of experiment, results still analyzed according to D-1.

3.4 Recommended seed re-treatment method

Overall, the best way to disinfect seeds is the one showing low infection rate without reducing germination rate. According to the data, in tomato lines, treatment b would be preferred, because only using low concentrate of sodium hypochloride could diminish most viroid, and have good germination rate. In pepper lines. Treatment f would be recommended for because using the same concentration of solution but soaking seeds for longer time could get better disinfection efficiency, in addition, the germination remain well.

Table 3 Infection rates of S*olanum lycopersicum* (tomato) and *Capsicum annuum* L. (pepper). Infection rate is divided into six ranks according to the ratio of infection. 0: 0%; 1: 20%; 2: 40%; 3: 60%; 4: 80%; 5: 100%.

	a	b	c	d	e	f	g	h
A	0	1	1	1	0	0	1	3
B	0	0	2	3	3	1	1	5
C	0	0	0	0	0	0	0	5
D	4	0	1	3	0	1	1	2
E	2	3	3	1	2	1	1	3

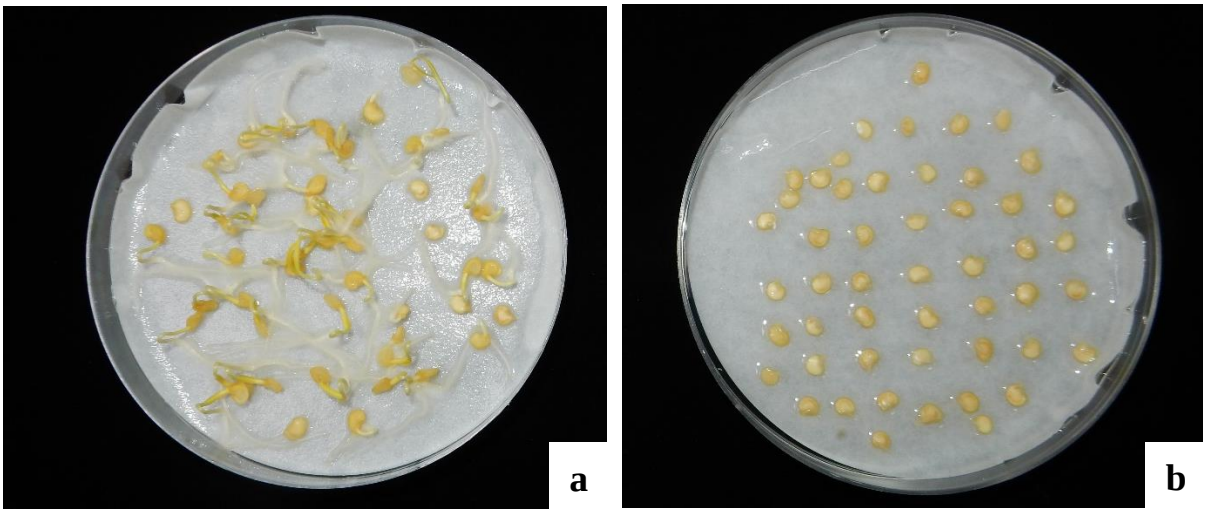

Fig. 3 Germination situation in line E at the seventh day. (a) f treatment (b) d treatment

Table 4 Germination rate of *Solanum lycopersicum* (tomato) and *Capsicum annuum* L. (pepper) at different treatments.

	a	b	c	d	e	f	g	h
A	0.24^{cd}	0.472^{ab}	0.538^{a}	0.428^{abc}	0.308^{bc}	0.233^{cd}	0.097^{d}	0.288^{bcd}
B	0.968^{a}	0.76^{a}	0.916^{a}	0.708^{a}	0.92^{a}	0.804^{a}	0.66^{a}	0.812^{a}
C	0.912^{ab}	0.904^{ab}	0.940^{a}	0.768^{c}	0.904^{ab}	0.872^{abc}	0.810^{bc}	0.912^{ab}
D	0.736^{a}	0.792^{a}	0.924^{a}	0.324^{b}	0.896^{a}	0.896^{a}	0.928^{a}	0.916^{a}
E	0.716^{bc}	0.816^{ab}	0.632^{c}	0^{d}	0.72^{abc}	0.852^{abc}	0.852^{a}	0.752^{ab}

Note: Data are expressed as mean which rounded to three decimal places with five replications. Different lowercase letters for each treatment of the same line indicate statistically significant different values ($p < 0.05$).

Table 5 Germination rates treated with different treatments in line D. D-1: original. D-2: repetitions with bad germination rate were removed. ($p<0.05$)

	a	b	c	d	e	f	g	h
D-1	0.736^{a}	0.792^{a}	0.924^{a}	0.324^{b}	0.896^{a}	0.896^{a}	0.928^{a}	0.916^{a}
D-2	0.736^{c}	0.792^{bc}	0.924^{a}	0.750^{bc}	0.896^{ab}	0.896^{ab}	0.928^{a}	0.916^{a}

Note: Data are expressed as mean which rounded to three decimal places with five replications. Different lowercase letters for each treatment of the same line indicate statistically significant different values ($p < 0.05$).

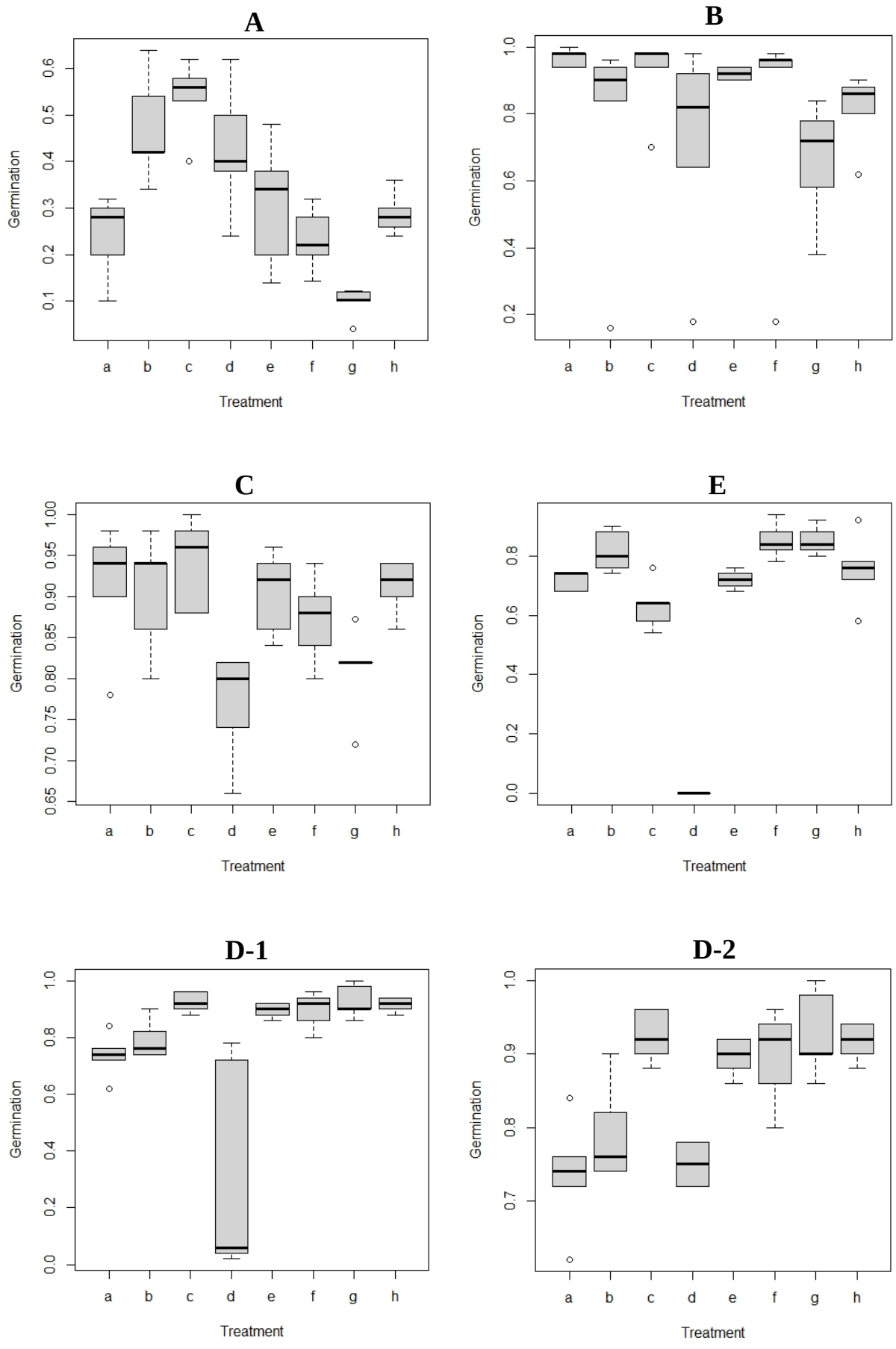

Fig. 4 Chart of germination rates treated with different treatments in per line. A: line A; B: line B; C: line C; E: line E. D-1: original. D-2: repetitions with bad germination rate were removed.

4. Discussion

This study found that there are some seeds remain infected after re-treatment. Viewing the result of infection rate, there are many situations like seeds treated with high concentration disinfectants but wouldn't get the better effect like using low concentration one. It is considered that the viroid infection level in the seeds was uneven. It might affect by viroid's species, the parts of seeds where viroid infected, or the degree of mixing seeds, etc. In line C, the infection rates were all zero in each treatment except for h treatment. It means that the infection level in line C might be low or the viroid type of line C (CLVd) can be easily removed. The seeds used in this experiment were produced many years ago, so the standard deviation of infection rate and germination rate are varied in some lines or between treatments. To solve the problem, seeds could be collected from same batch freshly produced, so that reproducibility and accuracy will be raised.

Observing the mean of germination rate in line D and line E, some of the values are not as high as tomato lines. In general, pepper seeds took more time to germinate, the germination rate couldn't be forecasted after doing seed re-treatment. Seeds in line D and E observed at the fourteenth day just grew radicles, so extend the germination test period may let the germination rate of treated pepper seeds be better than observed currently. In addition, seeds got moldy during the germination test because seeds were not remaining sterile when sowing into petri dishes. For this problem the experiment will be conducted in laminar airflow bench to reduce the situation of getting moldy.

There are some literatures discussing about viroid and the way how to diminish it last ten years. In this study, seeds re-treatment could remove viroid to an extent by NaOCl and HCl/TSP, besides seeds vigor are not be destroyed. There are some studies which mentioned other disinfectants could remove the viroid effectively. So the kind of disinfectant could be used next time. In addition, there are also some study about if the viroid could be transmitted from seeds to seedlings, so treated seeds could be sowed in seedling tray and checked if seedlings are infected by RT-PCR in the future. To summarize, seeds quarantine is still important for the world trading and other commercial activities, so it should be paid much attention on.

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