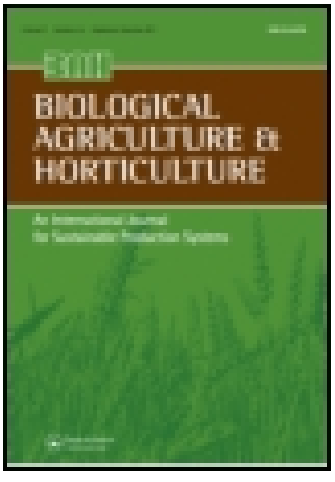

Biological Agriculture & Horticulture: An International Journal for Sustainable Production Systems

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbah20

Mycelium growth of early tomato blight pathogen, Alternaria solani, subjected to high dilution preparations

T.A. Modolon[a], P. Boff[b], M.I.C. Boff[a] & D.J. Miquelluti[a]

[a] Department of Agronomy, Santa Catarina State University - UDESC, CEP 88520-000, Lages, SC, Brazil

[b] Estação Experimental de Lages, EPAGRI - Agricultural Research and Rural Extension Agency of Santa Catarina State, Cx. Postal 191, CEP 88502-970, Lages, SC, Brazil

Published online: 30 Sep 2014.

To cite this article: T.A. Modolon, P. Boff, M.I.C. Boff & D.J. Miquelluti (2014): Mycelium growth of early tomato blight pathogen, Alternaria solani, subjected to high dilution preparations, Biological Agriculture & Horticulture: An International Journal for Sustainable Production Systems, DOI: 10.1080/01448765.2014.960451

To link to this article: http://dx.doi.org/10.1080/01448765.2014.960451

Biological Agriculture & Horticulture, 2014
http://dx.doi.org/10.1080/01448765.2014.960451

Mycelium growth of early tomato blight pathogen, *Alternaria solani*, subjected to high dilution preparations

T.A. Modolon[a], P. Boff[b]*, M.I.C. Boff[a] and D.J. Miquelluti[a]

[a]*Department of Agronomy, Santa Catarina State University – UDESC, CEP 88520-000 Lages, SC, Brazil;* [b]*Estação Experimental de Lages, EPAGRI – Agricultural Research and Rural Extension Agency of Santa Catarina State, Cx. Postal 191, CEP 88502-970 Lages, SC, Brazil*

(*Received 21 March 2013; accepted 28 August 2014*)

This study investigated the effect of high dilution preparations on the development of *Alternaria solani* mycelium, which causes early blight on tomato plants. Twelve bioassays were conducted under controlled conditions. Colony discs, 0.7 cm diameter, of *A. solani* were transferred into Petri dishes containing potato dextrose agar (PDA) or PDA + V8® vegetable juice media. High dilution preparations of *Arsenicum album*, *Nitricum acidum* and *Staphysagria* at 6, 12, 25, 30, 50, 60, 80 and 100 CH (centesimal Hahnemannian dilution scale) were applied either over the media or mixed in with it. Results showed that high dilution preparations have different effects on *A. solani* mycelium growth, according to the dynamization level. *A. solani* colonies were reduced by *A. album* 80 CH, by *N. acidum* 80 and 100 CH, and by *Staphysagria* 6, 30 and 60 CH compared with the control when applied over PDA medium. Higher mycelium reduction was observed in the PDA assays when the treatments were applied over the medium than when incorporated into it. The differences among high dilution treatments were distinctly greater on the PDA medium than on the PDA + V8®. Bioassay is a suitable method for screening high dilution preparations before studying them under field conditions.

Keywords: agro-homeopathy; agroecology; *Arsenicum album*; *Nitricum acidum*; *Staphysagria*

Introduction

Early blight, caused by the fungus *Alternaria solani* (Ellis & G. Martin) L.R. Jones & Grout, is an important destructive tomato disease that infects leaves, stalks and fruits, resulting in high yield losses. The pathogenic fungus survives on debris, and its resting spores and mycelia can infect other species of *Solanaceae* family such as potato, eggplant and paprika (Chaerani & Voorrips 2006). Low genetic resistance to pest and diseases in commercial tomato cultivars induces farmers, under conventional crop systems, to use a level of pesticides that does not meet consumer requirements (Modolon et al. 2012). The Brazilian sanitary agency Agência Nacional de Vigilância Sanitária (ANVISA) reported that more than 32.6% of tomato fruits sampled from the market had pesticide residues above the legal tolerance levels (ANVISA 2010).

It is therefore necessary to improve the tomato crop system with new ecological control strategies that would minimize hazardous effects to human health as well as to the environment (Diver et al. 1999). In general, farmers are willing to use such technologies if they are economically viable. The potential use of homeopathic preparations has been recently encouraged as an alternative pest management strategy, particularly for organic

*Corresponding author. Email: pboff@epagri.sc.gov.br

farming (Giesel et al. 2012; Modolon et al. 2012). Despite the limited information about the use of homeopathy in crops, it is well known that it has low cost, causes no residual effect and is rather simple to implement by farmers themselves (Boff 2008). In Brazil, homeopathic preparations for treating animal and plants are legally supported by Brazilian law no. 10.831, 23 December 2003 and regulated by no. 6.323, 27 December 2007, and IN 46/2011 of Brazilian Agriculture Ministry (Brasil 2011).

An extensive review by Scofield (1984) of experimental research in homeopathy concluded that it has potential value in the treatment and prevention of diseases in crops as well as animals and humans. Recently, the effect of homeopathic preparations on phytopathogenic fungus has been reported elsewhere (Betti et al. 2007; Toledo et al. 2011). Verma et al. (1969), in one of the earliest studies of homeopathy in plant pathology, aimed to control tobacco mosaic virus with *Lacchesis* and *Chimaphila* 200 CH (centesimal Hahnemannian dilution scale) and observed a reduction of 50% of the virus content in tobacco leaf discs. The severity of early blight on tomato plants in a greenhouse was reduced by applications of the *A. solani* biotherapic at 26, 27, 28 and 29 CH (Carneiro et al. 2010). Other homeopathic preparations described in the homeopathic materia medica for human beings were also reported to treat plants. Casali et al. (2009) found *Arsenicum album* to cause inhibition of fungus spore germination. *Nitricum acidum* has been shown to control cercosporiosis on plants (Bonato 2012). According to Betti et al. (2009), on the basis of 40 years of published research about homeopathic preparations applied to plant pathogens, it can be concluded that homeopathy is a promising technique for sustainable agriculture. Nevertheless, more research is necessary concerning dynamization levels (dilution and succession) and conditions reproducible under field conditions, which requires better experiment design with statistical analysis. In order to study the effect of high dilution preparations on plants under field conditions, it is necessary first to rank possible combinations preparation, application method and dose using screening bioassays should be designed (Modolon et al. 2012). The objective of this work, therefore, was to evaluate the effect of high dilutions of *A. album*, *N. acidum* and *Staphysagria* on mycelium growth of the tomato pathogenic fungus, *A. solani*.

Materials and methods

Experimental setup

The study was carried out in the Laboratory of Homeopathy and Plant Health, Lages Experimental Station, EPAGRI – Agriculture Research and Extension Service, Santa Catarina State, Brazil during 2009. Twelve bioassays, comprising the combinations of three homeopathic preparations with two media and two different methods of application, were performed in a climatic chamber at 22 ± 2°C, 70 ± 10% RH and a 16:08 h, light: dark photoperiod. Each bioassay was repeated three times. The homeopathic preparations tested were *A. album*, *N. acidum* and *Staphysagria* at 6, 12, 25, 30, 50, 60, 80 and 100 CH, prepared according to Farmacopéia Homeopática Brasileira (Brasil 1997). Sterilized water and non-intervention were the control treatments.

The original isolate of *A. solani* fungi was obtained from CNPH – Horticultural Research Centre and EMBRAPA – Brazilian Agricultural Research Corporation. The isolate was activated by transferring it into potato dextrose agar (PDA) medium and used to conduct the bioassays. The colonies were incubated for 10 days at 22 ± 2°C in chambers with diffuse light and then used for bioassays. Samples of the activated *A. solani* isolate were transferred to glass assay tubes in order to maintain a stock of fungus.

The experimental design was completely randomized with five repetitions for all of the 12 bioassays. All activities concerning installing bioassays, manipulations, and transferring media, fungus isolates and homeopathic preparations were done in a laminar flow hood. Experimental plots consisted of Petri dishes, 9 cm in diameter, in the centre of which a 0.7-cm-diameter disc of 10-day-old *A. solani* mycelia with PDA media was deposited with 20 ml of either PDA medium or PDA + V8®. Two media were prepared, PDA and PDA + V8® (a complex of eight vegetable juices), and sterilized. The homeopathic preparations were applied by dropping the solutions over the full colonized fungus disc or by adding them into the medium just before it was poured into the Petri dish.

High dilution preparation treatments

The high dilution preparations were centesimal dilutions (1:99) followed by 100 succussions (vertical angular movements) for each dilution level. That means they are identical to homeopathic preparations in such dynamization levels. The high dilution preparations were selected after comparing the symptoms of *A. solani* in tomato plants with analogous description of pathogenetic symptoms of the homeopathies, based on the Homeopathic Materia Medica (Vijnovsky 1980). This procedure was based on the Hahnemannian statement that says 'if homeopathic principals are validated than they could be applied to all living being' (Hahnemann 2001). The homeopathic matrices at 5 CH (fifth centesimal Hahnemannian dilution) were bought from a pharmacy in Lages, SC, Brazil and developed at the laboratory until the dynamization level used, as described in Farmacopéia Homeopática Brasileira (Brasil 1997). The homeopathic treatments and demineralized water (demi water) were prepared by a technician who labelled them by code, assuring a double-blind procedure for all treatments. The identity of the treatments was known only after statistical analysis.

The homeopathic dynamization protocol was as follows: 0.2 ml of 5 CH and 19.8 ml of alcohol at 5% were placed in a 30 ml amber bottle. The bottle was placed in a mechanic dynamizer (Autic®, Mod. Denise 10–20, Campinas, Brazil) and submitted to 100 succussions in order to obtain the 6 CH (sixth order of centesimal Hahnemannian dilution). The same procedure was repeated to obtain the 7 CH and the successive potencies until 100 CH. Demi water was used to dilute the alcohol to 5%. All activities until Petri dishes were sealed were done under a laminar flow hood. The dynamizations of 6, 12, 25, 30, 50, 60, 80 and 100 CH were used as the treatments. Demi water and non-intervention plots were control treatments. Either 100 μl of each treatment was spread over the mycelium disc in the Petri dish (application method 1) or 200 μl were incorporated into the media at 40°C, before solidification (application method 2). The Petri dishes were sealed with Parafilm® and incubated in a climatic chamber at 22 ± 2°C, 70 ± 10% RH and a 16:8 h light:dark photoperiod. The evaluation consisted of daily measurements of the diameter of mycelium growth on each Petri dish over 10 days.

Statistical analysis

Data from all bioassays were subjected to analysis of variance from a linear model with fixed effects (classical ANOVA model). The analysed variable was the diameter of mycelium growth. Data were submitted to Shapiro–Francia and Fligner–Killeen tests to check the normality and homogeneity of variances, respectively. Multiple comparisons of means were made by Tukey test at $p < 0.05$ significance.

To fill ANOVA assumptions, data were transformed by adding one (1) following the natural logarithm according to the model $y = \ln(x + 1)$. Statistical analysis was made with values in the logarithm scale and for the resulting means were applied the inverse transformations, $x = [\exp(y) - 1]$ to facilitate understanding by readers. The effect of the treatments on mycelium growth was considered among high dilution preparations along with the time after applying the preparations. All statistical analysis were performed by general linear model (GLM) and MIXED procedure of SAS® software (version 9.1) and software R (R Development Core Team 2008), with 5% significance level (Little et al. 2006).

Results and discussion

The mycelium growth of *A. solani* responded differently according to the dynamization level in the three studied high dilution preparations, *A. album*, *N. acidum* and *Staphysagria* (Tables 1–3). *A. album* in all dynamizations from 6 to 100 CH inhibited *A. solani* mycelium growth when applied over the mycelium disc of PDA media in comparison with demi water (Table 1). When *A. album* was incorporated into the PDA media, no effect on mycelium growth of *A. solani* was observed, except for the 12 CH dynamization that was lower and the 6 CH dynamization was higher in comparison to demi water. The treatment of demi water into the PDA medium gave higher mycelium growth than no-intervention probably because the water improves the medium for growing the fungus. Meanwhile, none of the high dilutions of *A. album*, either mixed into or over the enriched media (PDA + V8), inhibited the *A. solani* fungus in comparison with demi water. Increase in the dynamization level of *A. album* seemed to cause a nonlinear effect on *A. solani* mycelium growth. Some dynamizations of *A. album* incorporated into PDA media stimulated mycelium growth whereas others inhibited. This nonlinear effect of dynamization levels of high dilution preparations has been reported by other authors on

Table 1. Mycelium growth of *A. solani* colonies subjected to high dilution preparations of *A. album*.

	Diameter of mycelium growth (mm)			
	PDA*		PDA + V8®**	
High dilution	Over media	Into media	Over media	Into media
A. album 6 CH	19.5 cd	33.5 a	37.3 abc	36.4 ab
A. album 12 CH	20.1 c	25.8 cd	40.3 a	36.6 ab
A. album 25 CH	19.7 cd	28.7 bc	38.4 ab	36.1 ab
A. album 30 CH	22.6 bc	30.1 b	38.7 ab	37.6 a
A. album 50 CH	20.6 c	27.6 bcd	37.4 abc	37.9 a
A. album 60 CH	19.8 cd	29.8 b	39.4 ab	35.2 b
A. album 80 CH	17.1 d	28.4 bcd	34.9 c	36.6 ab
*A. album*100 CH	21.0 c	29.4 b	39.3 ab	37.5 a
Demi water	27.7 a	29.9 b	36.9 abc	36.7 ab
No-intervention	25.5 ab	25.6 d	36.3 bc	37.9 a
C.V. (%)**	7.46	16.18	12.42	8.41

Notes: Data are the average of five repetitions of 10-day evaluations and come from the three replications of bioassays per column. Means followed by the same letter in the column did not differ from each other by Tukey test ($p \leq 0.05$).
* PDA medium + V8® (vegetable juice).
** Coefficient of variance.

Table 2. Mycelium growth of *A. solani* colonies subjected to high dilution preparations of *N. acidum*.

	Diameter of mycelium growth (mm)			
	PDA*		PDA + V8®*	
High dilution	Over media	Into media	Over media	Into media
N. acidum 6 CH	11.3 ef	28.8 abc	44.0 abc	34.3 ab
N. acidum 12 CH	12.7 cd	31.6 a	44.9 ab	32.4 b
N. acidum 25 CH	13.0 c	29.6 abc	41.7 bc	33.2 ab
N. acidum 30 CH	11.7 ef	27.9 bc	47.2 a	32.3 b
N. acidum 50 CH	11.3 ef	30.4 ab	42.6 bc	32.5 b
N. acidum 60 CH	12.0 de	31.4 a	43.3 abc	35.8 a
N. acidum 80 CH	11.0 f	30.7 ab	44.5 abc	34.4 ab
N. acidum 100 CH	11.2 f	26.9 c	40.8 c	34.6 ab
Demi water	34.2 a	28.1 bc	44.3 abc	34.3 ab
No-intervention	28.8 b	28.7 abc	42.5 bc	34.4 ab
C.V. (%)**	10.42	16.64	11.45	11.51

Notes: Data are the average of five repetitions of 10-day evaluations and come from the three replications of bioassays per column. Means followed by the same letter in the column did not differ from each other by Tukey test ($p \leq 0.05$).
* PDA medium + V8® (vegetable juice).
** Coefficient of variance.

plants, suggesting that it is an oscillatory behaviour. Meanwhile, Bonato et al. (2009) observed increasing differences on the development of *Mentha arvensis* plants treated with *A. album* solutions at 6, 12, 24 and 30 CH dynamization compared with the control, but much less at 24 and 30 CH. Betti et al. (2007) observed an increase in the germination of wheat seeds after the application of *A. album* at 40, 42 and 45 DH (decimal dilution scale), but inhibition of germination at 35 DH.

Table 3. Mycelium growth of *A. solani* colonies subjected to high dilution preparations of *Staphysagria*.

	Diameter of mycelium growth (mm)			
	PDA*		PDA + V8®*	
High dilution	Over media	Into media	Over media	Into media
Staphysagria 6 CH	16.4 c	22.7 def	40.5 a	36.6 ab
Staphysagria 12 CH	16.8 bc	23.5 cdef	38.9 ab	33.5 c
Staphysagria 25 CH	17.3 bc	21.9 f	36.2 b	35.5 abc
Staphysagria 30 CH	16.3 c	29.3 a	40.4 a	36.9 ab
Staphysagria 50 CH	17.2 bc	25.2 cd	40.3 a	34.0 bc
Staphysagria 60 CH	15.9 c	24.4 cdef	38.4 ab	36.2 abc
Staphysagria 80 CH	17.1 bc	24.8 cde	37.7 ab	37.6 a
Staphysagria 100 CH	19.6 b	26.0 bc	40.6 a	36.1 abc
Demi water	29.8 a	22.4 ef	38.2 ab	34.1 bc
No-intervention	26.8 a	28.6 ab	38.7 ab	34.6 bc
C.V. (%)**	21.03	17.89	12.10	11.20

Notes: Data are the average of five repetitions of 10-day evaluations and come from the three replications of bioassays per column. Means followed by the same letter in the column did not differ from each other by Tukey test ($p \leq 0.05$).
* PDA medium + V8® (vegetable juice).
** Coefficient of variance.

N. acidum at all dynamizations studied, from 6 to 100 CH, caused inhibition of mycelium growth of *A. solani* when applied over the mycelium disc of PDA media (Table 2). On the other hand, *N. acidum* at 30 CH applied over the mycelium disc in PDA + V8® media stimulated *A. solani* mycelium growth in comparison to no-intervention but was not different from the demi water. Similarly, *N. acidum* at 12 and 60 CH, applied into PDA media, stimulated mycelium growth in comparison with demi water. This result contrasts with that of Saxena et al. (1987), who found that *N. acidum* at 200 CH inhibited 22 genera of fungi. According to Casali et al. (2009), *N. acidum* high dilution preparations were successfully used to treat fissures and ulcerations on fruits, flowers and stalk of tomato plants caused by cold wind. However, it can be noted that the effect of dynamization level is not linear.

All treatments which had *Staphysagria* dynamizations from 6 to 100 CH applied over PDA medium reduced *A. solani* mycelium growth in comparison with the control demi water (Table 3). *Staphysagria* at 25 CH applied into PDA medium reduced the fungus mycelium growth in comparison to the no-intervention control, but the treatment *Staphysagria* at 30, 50, 80 and 100 CH showed increasing in the mycelium growth of *Alternaria* when applied into media PDA and *Staphysagria* at 80 CH incorporated into PDA + V8®. Toledo et al. (2009) reported that *Staphysagria* at 100 CH reduced the mycelium growth of *A. solani*. In the Homeopathic Materia Medica, *Staphysagria* is described as a compound that has the properties to treat dilacerated tissues (Vijnovsky 1980). In this sense, it may be supposed that skin problems on tomato fruit, like those caused by *A. solani*, could be treated by using *Staphysagria*, but the dynamization level, as demonstrated in this study, does make a difference. None of the *Staphysagria* treatments had any mycelium reduction effect when applied either over or into the PDA + V8 media.

In general, higher growth of *A. solani* mycelium was observed in PDA + V8 medium than in PDA only. On the other hand, higher inhibition of the fungus was observed in the treatments where applications were applied over the mycelium disc that had previously been put on PDA than where the treatment was incorporated into the medium. The basic medium, PDA, was found to be more suitable for screening high dilution preparations than the PDA + V8 medium (Table 3). However, further field studies are needed before any conclusions can be made as to how to implement homeopathic therapy for tomato crops under field conditions.

A nonlinear response of fungus mycelium growth to high dilution applications agrees with Casa et al. (2007) who observed a nonlinear biomass accumulation of willow (*Salix viminalis*) related to high dilution applications. Likewise, da Silva et al. (2012) also argued that this tendency is influenced by the rhythmic movements in nature, since the mode of action of high dilution preparations is in the vital body of the living system. Also, this response may follow the similarity between the symptoms that high dilution preparations cause and the symptoms of the organism to be treated, in other words, the cure by similar diseased effect, which is the first principle of homeopathy. Also, it seems that the exposure method of fungus mycelium to high dilution preparations may play an important role for screening potential dynamizations.

References

ANVISA. 2010. Programme for analysis of pesticide residues in food-PARA; activities report of the 2009. Brasília: Gerência Regional de Toxicologia. [In Portuguese].

Betti L, Trebbi G, Lazzarato L, Fantino MG, Nani D. 2007. Effects of homeopathic dilutions on plants and the potential use of homeopathy on plant diseases. Fitopatol Bras. 32:75–77.

Betti L, Trebbi G, Majewsky V, Scherr C, Shah-Rossi D, Jager T, Baumgartner S. 2009. Use of homeopathic preparations in phytopathological models and in field trials: a critical review. Homeopathy. 98:244–266.
Boff P. 2008. Healthy agriculture: from prevention of diseases, pests and parasites to no-residual therapy. Lages: Epagri-Udesc. [In Portuguese].
Bonato CM. 2012. Simple homeopathy: an alternative for family farmers. 3rd ed. Maringa: UEM-CAPA.
Bonato CM, Proença GT, Reis B. 2009. Homeopathic drugs *Arsenicum album* and *Sulphur* affect the growth and essential oil content in mint (*Mentha arvensis* L.). Acta Sci Agron. 31:101–105.
Brasil. 1997. Brazilian homeopathic pharmacopeia. 4th ed. São Paulo: Atheneu. [In Portuguese].
Brasil. 2011. Normative instruction n. 46 of the 6th October 2011. Diário Oficial da República Federativa do Brasil. Brasília: Ministério da Agricultura, Pecuária e do Abastecimento. [In Portuguese].
Carneiro SMTPG, Romano EDB, Pignoni E, Teixeira MZ, Costa Vasconcelos ME, Gomes JC. 2010. Effect of biotherapic of *Alternaria solani* on the early blight of tomato-plant and the *in vitro* development of the fungus. Int J High Dilution Res. 9:147–155.
Casa J, Boff MIC, Rech TD, Boff P. 2007. Salix morphogenesis after treatment with homeopathic and phytotherapic preparations. Agropecu Catarin. 20: 71–74. [In Portuguese].
Casali VWD, de Andrade FMC, Duarte ESM. 2009. Acology of high dilutions. Viçosa: UFV–Departamento de Fitotecnia. [In Portuguese].
Chaerani R, Voorrips RE. 2006. Tomato early blight (*Alternaria solani*): the pathogen, genetics, and breeding for resistance. J Gen Plant Pathol. 72:335–347.
da Silva HA, Parizotto AV, Moreira FC, Marques RM, Reis B, Bonato CM. 2012. The effect of high dilutions of *Pulsatilla nigricans* on the vigour of soybean seeds subjected to accelerated aging. Acta Sci Agron. 34:201–206.
Diver S, Kuepper G, Born H. 1999. Organic tomato production. ATTRA horticulture production guide. Butte (MT): National Center for Appropriate Technology.
Giesel A, Boff MIC, Boff P. 2012. The effect of homeopathic preparations activity level of *Acromyrmex* leaf-cutting ants. Acta Sci Agron. 34:445–451.
Hahnemann S. 2001. Organon of the healing art. 6th ed. São Paulo: Robe. [In Portuguese].
Little RC, Milliken GA, Stroup WW, Wolfinger RD, Schabenberger O. 2006. SAS® for mixed models. Cary (NC): SAS Institute Inc.
Modolon TA, Boff P, Boff MIC, Miquelluti DJ. 2012. Homeopathic and high dilution preparations for pest management to tomato crop under organic production system. Hortic Bras. 30:51–57.
R Development Core Team. 2008. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing. [cited 2010 Mar 1]; Available from: http://www.R-project.org
Saxena A, Pandey ML, Gupta RC. 1987. Effect of certain homeopathic drugs on incidence of seed-borne fungi and seed germination of *Abelmoschus esculentus*. Indian J Mycol Plant Pathol. 17:191–192.
Scofield AM. 1984. Experimental research in homeopathy – a critical review. Br Homoeopath J. 73:161–180.
Toledo MV, Stangarlin JR, Bonato CM. 2009. The use of homeopathic preparations of sulphur and ferrum sulphuricum to control early-blight in tomato. Rev Bras Agroecol. 4:475–478. [In Portuguese].
Toledo MV, Stangarlin JR, Bonato CM. 2011. Homeopathy for the control of plant pathogens. In: Méndez-Vilas A, editor. Science against microbial pathogens: communicating current research and technological advances. Badajoz: Formatex Research Center; p. 1063–1067.
Verma HN, Verma GS, Verma VK, Krishna R, Srivastava KM. 1969. Homeopathic and pharmacopeia solutions as inhibitors of tobacco mosaic virus. Indian Phytopathol. 22:188–193.
Vijnovsky B. 1980. Treatise of homeopathic materia medica. Vols. 1–3. Rio de Janeiro: Editora: Mukunda. [In Portuguese].