Faridi et al. | J Pure Appl Microbiol | 16(2):943-949 | June 2022
Article 7531 | https://doi.org/10.22207/JPAM.16.2.16
Published Online April 30, 2022
Print ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

First Report of Physico-Chemical Agents of Viable But Non Culturable (VBNC) forms of *Ralstonia pseudosolanacearum*

Neha Faridi[1,2], Merwyn Samuel[1]*, Shalini Bhatt[1], Ankur Agarwal[1], Veena Pande[2] and Madhu Bala[1]

[1]Defence Institute of Bio-Energy Research (DIBER), DRDO, P.O Arjunpur, Haldwani - 263 139, Uttarakhand, India.
[2]Department of Biotechnology, Bhimtal, Kumaun University, Nainital - 263 001, Uttarakhand, India.

Abstract

The germicidal efficacy of various physico-chemical agents against culturable bacterial cells and bacterial spores is well studied and reviewed; except for the dormant Viable But Non Culturable state (VBNCs). VBNC is a special physiological state, where the bacterium is not culturable in culture media, but remains alive, infective and virulent. The aim of this study is to evaluate the germicidal efficacy of three physico-chemical agents against the VBNC forms of destructive phytopathogen *Ralstonia pseudosolanacearum.* Effect of thermotherapy Formalin and Sodium Hypochlorite on these forms was evaluated using standard plate count and reverse culturability tests. The disinfectants were found equally effective against the culturable cells and VBNC forms at recommended concentrations.

Keywords: VBNC, Stress, *Ralstonia pseudosolanacearum,* Bacterial wilt

***Correspondence:** merwyn@diber.drdo.in

(Received: January 6, 2022; accepted: February 15, 2022)

Citation: Faridi N, Samuel M, Bhatt S, Agarwal A, Pande V, Bala M. First Report of Physico-Chemical Agents of Viable But Non Culturable (Vbnc) forms of *Ralstonia pseudosolanacearum*. *J Pure Appl Microbiol.* 2022;16(2):943-949. doi: 10.22207/JPAM.16.2.16

INTRODUCTION

Ralstonia pseudosolanacearum (Previously *R. solanacearum* Phylotype 1 & 3) is a soil, seed and waterborne bacterial phytopathogen which causes the bacterial wilt disease across 53 different botanical families.[1,2] The bacterium infects through the root epidermis and colonizes the xylem, resulting in wilting and subsequent plant death. In the environment, the bacterium survives in reservoir plants, soil and water through diverse strategies, including the formation of Viable But NonCulturable (VBNC) state.[3]

Many bacterial species are known to enter the VBNC state during the logarithmic growth phase (stochastic transformation) and stressed phase triggered by chemical and environmental factors. Relative to culturable cells, VBNC forms possess higher physical and chemical tolerance against low salinity, pH, ethanol, chlorine and antibiotics.[4-8] *R. pseudosolanacearum* enters into VBNC state under nutrient starvation, low temperature and heavy metal exposure and are capable of causing disease outbreaks when environmental conditions turn favourable for infection.[9-11] *R. pseudosolanacearum* is a soil, water and seed-borne pathogen.[12] To reduce its load in seeds and other planting materials, laboratories perform various phytosanitary measures like thermotherapy at 60°C for two hours[13] treatment with 50% sodium hypochlorite[14] and 0.5% formalin.[15] The efficacy of these chemical and physical agents on culturable cells of different microcosms have been extensively published and validated through laboratory protocols. But, to our knowledge, no scientific publication has reported the efficacy of the above mentioned phytosanitary measures and disinfectants on VBNC forms of *R. pseudosolanacearum* or in any other phytopathogen. In this study, we have evaluated the germicidal efficacy of physico-chemical agents on two strains of *R. pseudosolanacearum* culturable cells and VBNC forms.

MATERIALS AND METHODS

Preparation of microcosms

R. pseudosolanacearum isolates used in this study are DIBER115 (NCBI Accession number MG266193) and DIBER118 (NCBI Accession number MG266203). These isolates collected from different locations in the state of Uttarakhand, India was confirmed by 16s rDNA sequencing and pathogenicity assays. The phylotype of the isolates were found to be Phylotype I of *R. solanacearum* species complex[16] which are taxonomically reclassified as *R. pseudosolanacearum*.[1] The other strains used in this study were *Ralstonia pickettii* (ATCC 648) and *E. coli* (ATCC 10536). *R. pseudosolanacearum* strains were grown and counted in mSMSA Modifed Semi Selective South Africa Media (mSMSA) broth and mSMSA plates, respectively.[17] Other strains were grown in Nutrient broth and Nutrient agar. Dead cells of *R. pseudosolanacearum* (DIB115D and DIB11 8D) for controls were prepared by exposing live cells in logarithmic phase with UV at 2,000 µJ/cm^2. For all Physico-Chemical treatments, the concentration of culturable cells and VBNC forms were maintained between 1-5X10^6 Cfu/ml. For concentration of VBNC forms, was fixed after counting the resuscitated cells.

Preparation and confirmation of *R. pseudosolanacearum* VBNC forms

VBNC forms of *R. pseudosolanacearum* (DIB115V and DIB118V) were prepared by incubating water washed cells at 4°C in a refrigerator for 180 days.[18] Generated VBNC forms were confirmed by direct viable count and reverse culturability assay.[19] Before proceeding for physical and chemical treatments, the VBNCs were normalised to a concentration of 1-5X10^6 Cfu/ml using sterile water.

The data presented in this study are the means of two experiments. The difference between the samples duplicates were analysed by two-tailed "Student's t" test. P-Value ≤ 0.05 denotes significant variation between the sample duplicates.

Physical and Chemical Treatment

Thermotherapy was carried out at 60°C for 2 hrs by placing 1 ml of microcosms in glass test tubes in a heating water bath as per the protocol given by Grondeau and coworkers. After treatment, the viability, reverse culturability and Pathogenicity index were determined after washing the microcosms twice with sterile distilled water.

For Chemical treatment, pelleted microcosms were exposed to different v/v concentrations of Formalin and NaOCl. Sauer and Burroughs in 1986 noticed the differences in disinfection efficacy among different brands of NaOCL. Hence to simplify the concentration of NaOCl to a universal quantity for Comparision, we diluted the NaOCL to different v/v concentrations and determined the active Cl- concentration.[20-21] The chemical agents were allowed to react with microcosms for 5 minutes and 10 minutes. After physical and chemical treatments, the microcosms were washed twice with sterile distilled water, thereafter processed for viability and reverse culturability assay and pathogenicity determination. Appropriate controls were also taken.

Reverse Culturability Assay

Reverse culturability otherwise known as resuscitation is used to confirm VBNC forms. All stress exposed Microcosms were resuscitated[22] in sterile distilled water supplemented with 1000u/ml of catalase (HiMedia Labs, Mumbai). Resuscitation was carried at 28°C for five days and the resultant growth was counted on mSMSA plates after washing twice with sterile distilled water.

Pathogenicity assay

Pathogenicity was performed on bacterial wilt susceptible Tomato variety seeds ICAR H-86 as per the protocol developed by Singh groups[23] The disease severity scale was determined on 7th day according to the key: 0 = No symptoms; 1 = One leaf wilted; 2 = Two leaves wilted; 3= Plant dead. The Disease index (DI) was calculated by the formula DI = DI=ΣRT×100/4N. Where: R = disease severity scale (0, 1, 2, 3); T = number of wilted plants in each category and N = total number of tested plants. Pathogenicity tests were not performed for *E. coli* and *R. pickettii.*

RESULTS

Confirmation of VBNC state in *R. pseudosolanacearum*

In sterile water, *R. pseudosolanacearum* strains DIBER115 and DIBER118 lost their culturability within 180 days as determined by direct plate counting. Hence, the 'Alive' statuses of the cells were confirmed by reverse culturability, where about 80-90% of the cells

Table 1. Formation of VBNCs of *R. pseudosolanacearum* strains in psychrophilic stress

		0	60	120	180	240	300	360	420	480
DIBER115	DVC	8.01±0.22 (P=0.08)	7.40±0.24 (P=0.57)	4.64±0.45 (P=0.67)	0	0	0	0	0	0
	RC	8.03±0.02 (P=0.23)	8.19±0.051 (P=0.19)	8.68±0.12 (P=0.69)	8.29±0.241 (P=0.47)	8.08±0.01 (P=0.169)	3.35±0.027 (P=0.457)	0	0	0
	PI	100	58.3	60	50.5	45.5	61.5	65.3	73.3	33.3
DIBER118	DVC	8.32±0.01 (P=0.20)	8.33±0.10 (P=0.90)	6.846±0.007 (P= 0.79)	0	0	0	0	0	0
	RC	8.41±0.01 (P=0.8)	8.19±0.01 (P=0.2)	8.64±0.16 (P=0.8)	8.28±0.31 (P=0.5)	7.36±0.01 (P=0.4)	3.37±0.03 (P=0.5)	0	0	0
	PI	100	93.3	60	73.3	60	33.3	33.3	33.3	40

DVC - Direct Plate Count; RC- Reverse Culturability; PI-Pathogenicity Index; PI is expressed in percentage and DVC and RC were expressed in Log Cfu/ml.

Table 2. Effect of Chemicals and Thermotherapy on Microcosms

		Before Treatment	Active Cl. Conc (%) 5mins				Formaldehyde (%) 5mins			Thermo-therapy 2hrs
			0.5	0.09	0.05	0.035	0.5	0.25	0.1	0
DIBER115	DVC	6.39±0.002 (P=0.51)	0	0	0	0	0	0	0	0
	RC	6.44±0.43 (P=0.75)	0	0	0	0	0	0	0	0
	PI	100	0	0	0	0	0	0	0	0
DIB115V	DVC	0	0	0	0	0	0	0	0	0
	RC	6.25±0.08 (P=0.28)	0	0	0	0	0	0	0	0
	PI	50	0	0	0	0	0	0	0	0
DIBER118	DVC	6.28± 0.35 (P=0.10)	0	0	0	0	0	0	0	0
	RC	6.34± 0.034 (P=0.33)	0	0	0	0	0	0	0	0
	PI	100	0	0	0	0	0	0	0	0
DIB118V	DVC	0	0	0	0	0	0	0	0	0
	RC	6.13±0.11 (P=0.29)	0	0	0	0	0	0	0	0
	PI	40	0	0	0	0	0	0	0	0
E. coli	DVC	6.37±0.039 (P=0.36)	0	0	0	0	0	0	0	6.42±0.09 (P=0.052)
	RC	6.38±0.099 (P=0.37)	0	0	0	0	0	0	0	6.44±0.09 (P=0.5)
R.pickettii	DVC	6.28±0.055 (P=0.32)	0	0	0	0	0	0	0	5.98±0.044 (P=0.07)
	RC	6.35±0.58 (P=0.41)	0	0	0	0	0	0	0	5.96±0.04 (P=0.5)

DVC- Direct Plate Count; RC- Reverse Culturability; PI-Pathogenicity Index; PI is expressed in percentage and DVC and RC were expressed in Log Cfu/ml.

were resuscitated following catalase treatment (Table 1). This confirms that a substantial culturable microcosms has transformed into VBNC forms. The VBNC forms were rechristened as DIB115V and DIB118V. Hereafter, experiments were performed to determine the germicidal efficacy of physico-chemical agents against *R. pseudosolanacerum* cells and its VBNC forms.

Efficacy of Physical Treatment

The Phytosanitary method evaluated was thermotherapy, which was performed at 60°C for 2 hrs. The prescribed temperature and time were found sufficient for total inactivation of the pathogen as revealed by direct plate count, reverse cultivability, pathogenicity tests. Meanwhile, some culturable cells of *E. coli* and *R. pickettii* cells showed resistance towards thermotherapy (Table 2).

Effect of Chemical Treatment

All the chemical disinfectants at concentrations recommended for phytosanitation were effective in inactivating all the culturable and VBNC microcosms within 5 minutes. None of the treated microcosms were successful in causing bacterial wilt in host plants. The effect of different concentrations of formalin and NaOCl is summarized in (Table 2).

DISCUSSION

To prevent seed-borne infections, various phytosanitary measures like thermotherapy, washing with chemical disinfectants like Sodium Hypochlorite (NaOCl) and formalin 15 are widely used. In this work, we evaluated the efficacy of these phytosanitary measures. Cells that are metabolically or physiologically active (Viable) but cannot be cultured on microbial media are known as Viable But Nonculturable Cells (VBNC). To confirm the live/dead status of microcosms, we used direct plate count and reverse culturability (plate count after resuscitation) with catalase, as reported.[24]

VBNC forms of two *R. pseudosolanacearum* strains were prepared by incubating culturable cells at refrigerating temperature for 180 days. The transformation was periodically monitored by direct plate count and reverse culturability assay. Both the strains DIBER115 and DIBER118 reached non-culturable state in 180 days. The fundamental property of VBNC forms is resuscitation.[25] To confirm VBNC transformation on 180th day, reverse culturability assay using catalase was performed. In 180 days, 90% of the cells were reverse culturable. Previous studies with Log 6.78 *R. solanacearum* cells have reported loss of pathogenicity within 125 days.[18] This variation between ours and Leo et al is due to strain to strain variation.[26]

Thermotherapy, at 60°C for 2 hrs is widely followed approach for phytosanitation. The approximate maximal growth temperature of *R.pseudosolanacearum* is 37-39°C with optimum growth temperature between 27–35°C.[27] Increasing the temperature beyond the optimal value hampers the cells survivability. Thermotherapy to inactivate pathogens depends on incubating microcosms at temperatures beyond the maximal growth temperature.[13] Previously, a 7 log reduction of *R. solanacearum* culturable cells in infested soil using thermotherapy at 60°C for 2 hrs was achieved.[28] We are of the view, that the effect of heat on culturable cells as revealed by Jirasak and Siricha should not be generalized to VBNC forms. Hence, we exposed culturable cells and VBNC forms to 60°C for 2 hrs and observed total elimination of *R. pseudosolanacearum* culturable cells and VBNC forms as confirmed by zero colony counts (Table 2).

Sodium hypochlorite is one of the best Chlorine releasing agents, which forms hypochlorite ion, OCl- and hypochlorous acid (HOCl), which are known oxidizers of various biomolecules.[20] In our laboratory, we routinely treat tomato seeds with 50% v/v NaOCl (which corresponds to 0.5% of active Chlorine) for 10 minutes and achieve seed germination efficacy of >90%. When exposed to 0.5% of active Chlorine, all microcosms, culturable cells and VBNCs were totally inactivated within 5 mins of exposure. To further investigate the effect at lower concentrations, NaOCl was diluted to active chlorine concentrations of 0.1%, 0.05% and 0.035% (0.035% of active Chlorine equals 10% v/v NaOCl). Previous studies with NaOCl[29] have established that, disinfection of seeds is incomplete at 5% v/v of NaOCl, hence we didn't continue with concentrations below 10% v/v dilutions. In this study NaOCl, was found effective upto 0.035% of active chlorine. Hence, it can be confirmed that, NaOCl can effectively sanitize seeds contaminated with *R. pseudosolanacearum* cells and VBNC forms at active chlorine concentration above 0.05%.

We noticed the same disinfection with formalin as in NaOCl. At concentrations used for seed sanitization (0.5% v/v), all microcosms were inactivated within 5 mins of exposure. This trend was also observed at treatments below 0.25% v/v. It was observed that unlike thermotherapy, both the chemical disinfectants didn't discriminate between the studied microcosms. No CFU count indicative of resuscitation was observed at tested concentrations, indicating that NaOCl with active chlorine concentration between 0.5-0.035% and formalin concentration between 0.5-0.25% can be used for germplasm sanitization within 5 minutes. However, it is recommended to treat the seeds for 10 minutes.

CONCLUSIONS

The germicidal action of phytosanitary methods against culturable cells has been described and endorsed previously. In this work, we evaluated the efficacy of phytosanitation method against VBNC. It was observed that the methods are as equally effective against VBNC as for culturable cells.

ACKNOWLEDGMENTS

The authors would like to thank Director DIBER, DRDO Haldwani and Co-ordinator, Department of Biotechnology, Bhimtal, Kumaun University, Nainital, India for providing the facilities, support and encouragement.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

ETHICS STATEMENT

Not applicable.

DATA AVAILABILITY

All data sets generated or analyzed during this study are included in the manuscript.

REFERENCES

1. Safni I, Cleenwerck I, De Vos P, Fegan M, Sly L, Kappler U. Polyphasic taxonomic revision of the *Ralstonia solanacearum* species complex: proposal to emend the descriptions of *Ralstonia solanacearum* and *Ralstonia syzygii* and reclassify current *R. syzygii* strains as *Ralstonia syzygii* subsp. *syzygii* subsp. nov., *R. solanacearum* phylotype IV strains as *Ralstonia syzygii* subsp. *indonesiensis* subsp. nov., banana blood disease bacterium strains as *Ralstonia syzygii* subsp. *celebesensis* subsp. nov. and *R. solanacearum* phylotype I and III strains as *Ralstonia pseudosolanacearum* sp. nov. *Int J Syst Evol Microbiol.* 2014;64(9):3087-3103. doi: 10.1099/ijs.0.066712-0
2. Hayward AC, Hartman GL. Bacterial wilt: the disease and its causative agent, *Pseudomonas solanacearum.* In Bacterial wilt: the disease and its causative agent, *Pseudomonas solanacearum.* CAB International, Wallingford. 1994.
3. Alvarez B, Biosca EG, Lopez MM. On the life of *Ralstonia solanacearum,* a destructive bacterial plant pathogen. *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*. 2010;1:267-279.
4. Oliver JD. Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol Rev.* 2010;34(4):415-425. doi: 10.1111/j.1574-6976.2009.00200
5. del Mar Lleo M, Benedetti D, Tafi MC, Signoretto C, Canepari P. Inhibition of the resuscitation from the viable but non-culturable state in *Enterococcus faecalis. Environ Microbiol*. 2007;9(9):2313-2320. doi: 10.1111/j.1462-2920.2007.01345.x
6. Wong HC, Wang P. Induction of viable but nonculturable state in *Vibrio parahaemolyticus* and its susceptibility to environmental stresses. *J Appl Microbiol.* 2004;96(2):359-366. doi: 10.1046/j.1365-2672.2004.02166
7. Rowe MT, Dunstall G, Kirk R, Loughney CF, Cooke JL, Brown SR. Development of an image system for the study of viable but non-culturable forms of *Campylobacter jejuni* and its use to determine their resistance to disinfectants. *Food Microbiol.* 1998;15(5):491-498. doi: 10.1006/fmic.1997.0178
8. Weichart D, Kjelleberg S. Stress resistance and recovery potential of culturable and viable but nonculturable cells of *Vibrio vulnificus. Microbiology.* 1996;142(4):845-853. doi: 10.1099/00221287-142-4-845
9. Kong HG, Bae JY, Lee HJ, et al. Induction of the viable but nonculturable state of *Ralstonia solanacearum* by low temperature in the soil microcosm and its resuscitation by catalase. *PLoS One.* 2014;9(10):e109792. doi: 10.1371/journal.pone.0109792
10. Alvarez B, Lopez MM, Biosca EG. Survival strategies and pathogenicity of *Ralstonia solanacearum* phylotype II subjected to prolonged starvation in environmental water microcosms. *Microbiology.* 2008 ;154(11):3590-3598. doi: 10.1099/mic.0.2008/019448-0
11. Elsas JD, Kastelein P, de Vries PM, van Overbeek LS. Effects of ecological factors on the survival and physiology of *Ralstonia solanacearum* bv. 2 in irrigation water. *Can J Microbiol.* 2001;47(9):842-854. doi: 10.1139/w01-084
12. Dey P, Hossain I, Hossain MD. Isolation and identification of seed borne *Ralstonia solanacearum* from tomato and brinjal in Bangladesh. *J Agric. Vet. Sci.* 2017;10:32-39.
13. Grondeau C, Samson R, Sands DC. A review of thermotherapy to free plant materials from pathogens, especially seeds from bacteria. *Critical Reviews in Plant Sciences.* 1994;13(1):57-75. doi: 10.1080/07352689409701908
14. Sauer DB, Burroughs R. Disinfection of seed surfaces with sodium hypochlorite. *Phytopathology.* 1986;76(7):745-749. doi: 10.1094/Phyto-76-745
15. Atwood WM. Physiological studies of effects of formaldehyde on wheat. *Botanical Gazette*. 1922;74(3):233-263. doi: 10.1086/333096
16. Prior P, Fegan M. Diversity and molecular detection of *Ralstonia solanacearum* race 2 strains by multiplex PCR. Bacterial wilt disease and the *Ralstonia solanacearum* species complex. 2005:405-414.
17. Elphinstone JG, Hennessy J, Wilson JK, Stead DE. Sensitivity of different methods for the detection of *Ralstonia solanacearum* in potato tuber extracts. *Eppo Bulletin.* 1996;26(3-4):663-678. doi: 10.1111/j.1365-2338.1996.tb01511
18. van Overbeek LS, Bergervoet JH, Jacobs FH, van Elsas JD. The low-temperature-induced

viable-but-nonculturable state affects the virulence of *Ralstonia solanacearum* biovar 2. *Phytopathology.* 2004;94(5):463-469. doi: 10.1094/PHYTO.2004.94.5.463

19. Lahtinen SJ, Ahokoski H, Reinikainen JP, et al. Degradation of 16S rRNA and attributes of viability of viable but nonculturable probiotic bacteria. *Lett Appl Microbiol.* 2008;46(6):693-698. doi: 10.1111/j.1472-765X.2008.02374
20. McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action, and resistance. *Clin Microbiol Rev.* 1999;12(1):147-179. doi: 10.1128/CMR.12.1.147
21. Willson VA. Determination of available chlorine in hypochlorite solutions by direct titration with sodium thiosulfate. *Ind Eng Chem Anal Ed.* 1935;7(1):44-45. doi: 10.1021/ac50093a022
22. Kong IS, Bates TC, Hulsmann A, Hassan H, Smith BE, Oliver JD. Role of catalase and oxyR in the viable but nonculturable state of *Vibrio vulnificus. FEMS Microbiology Ecology.* 2004;50(3):133-142. doi: 10.1016/j.femsec.2004.06.004
23. Singh N, Kumar R, Ray SK. An innovative approach to study *Ralstonia solanacearum* pathogenicity in 6 to 7 days old tomato seedlings by root dip inoculation. *Bio-protocol*. 2018;8(21):e3065. doi: 10.21769/BioProtoc.3065
24. Zhao X, Zhong J, Wei C, Lin CW, Ding T. Current perspectives on viable but non-culturable state in foodborne pathogens. *Front Microbiol.* 2017;8:580. doi: 10.3389/fmicb.2017.00580
25. Kim JS, Chowdhury N, Wood TK. Viable but non-culturable cells are persister cells. *BioRxiv.* 2017. doi: 10.1101/186858
26. Ramamurthy T, Ghosh A, Pazhani GP, Shinoda S. Current perspectives on viable but non-culturable (VBNC) pathogenic bacteria. *Front Public Health.* 2014;2:103. doi: 10.3389/fpubh.2014.00103
27. Kelman A. The bacterial wilt caused by *Pseudomonas aolanacearum.* A literature review and bibliography. *N Carolina Agric Exp Stn Tech Bull.* 1953;99:194.
28. Kongkiattikajorn J, Thepa S. Increased tomato yields by heat treatment for controlling *Ralstonia solanacearum* in soil. *Agriculture and Natural Resources.* 2007;41(5):219-224.
29. Himabindu KB, Shanthi Priya M, Reddy DM, et al. Studies on the effect of various sterilants and culture conditions on invitro seed germination in tomato (*Solanum lycopersicum*), *International Journal of Applied Biology and Pharma ceutical Technology*. 2012; 3(4):47 6-480.