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Isolation and identification of five alcohol-defying *Bacillus* spp. covertly associated with *in vitro* culture of seedless watermelon

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Five distinct bacterial clones (3 × WMARB-1 to 5) were isolated from the spent alcohol used for tool-disinfection during the subculturing of apparently clean, long-term micropropagated triploid watermelon (*Citrullus lanatus* L.) cultures that harboured bacteria in covert form. The isolates belonged to aerobic, Gram-positive, endospore-forming bacilli. Four of these were identified as *Bacillus fusiformis* (3 × WMARB-2), *B. pumilus* (3 × WMARB-3), *B. subtilis* (3 × WMARB-4) and *B. flexus* (3 × WMARB-5) through 16S rDNA sequence analysis (approx. 1450 bp), while isolate 3 × WMARB-1 was identified through fatty acid profiling as *B. megaterium*. These as well as other spore-forming organisms that were employed as control (*B. thuringiensis* and *Brevibacillus* sp.) showed survival in 70% or absolute alcohol from overnight to several days, while non-spore forming checks, including Gram-negative *Escherichia coli*, *Pantoea*, *Sphingomonas*, *Agrobacterium* spp. and Gram-positive *Microbacterium* sp. were killed within a few minutes. The alcohol tolerance property of *Bacillus* spores proved to be a threat to plant tissue cultures owing to the likelihood of unsuspected lateral spread of contamination through inadequately flamed tools when alcohol is used as a sterilant, compounded by their covert survival in tissue-culture medium, and in general microbiology, wherever alcohol is used as a surface disinfectant.

Keywords: *Citrullus lanantus*, endospore resistance, fatty acid profiling, microbial contamination, plant tissue culture.

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ALCOHOL-dip and flaming is often employed during plant and animal tissue cultures and microbial culture transfers for disinfection of culture-handling tools between successive samples. Three alcohols are generally used as disinfectants, namely methanol, ethanol and isopropanol[1]. The bactericidal value of alcohols increases with chain length or molecular weight, and thus isopropyl alcohol is the most effective among the three[1–3]. Ethanol, however, is the most widely used alcohol. Alcohols readily inactivate the vegetative bacterial cells through protein denaturation and membrane leakage, but are generally ineffective against spores[1,4,5]. Flaming, which supplements the alcohol action, is expected to eliminate any cell that survives alcohol challenge. Absolute alcohol is relatively less effective as a sterilant due to low penetrability. Water increases its wetability[1,4,5] with maximum disinfection activity around 70–80% v/v. At this concentration, alcohol would not give a good flame. For all practical purposes absolute alcohol or the single distillation product of alcohol-fermentation from molasses, namely rectified or surgical spirit (96% ethanol), which is more economical, is widely used for surface sterilization or for alcohol-flaming.

Plant and animal cell cultures are expected to be free from all microbes, and asepsis is a primary requirement for tissue culture work[6–8]. While dealing with micropropagation of a value-added triploid seedless watermelon[9], we have encountered the covert association of a series of bacteria after years of continuous *in vitro* culturing, which was identified as the cause for decline in the performance of cultures[10]. Eight different organisms comprising four Gram-positive (*Bacillus* spp.) and four Gram-negative (three *Pseudomonas* spp. and one *Aeromonas* sp.) strains were isolated from these cultures[10]. These organisms seldom expressed on tissue culture medium, and it warranted an indexing of medium and/or tissue using bacteriological medium for their detection[10,11]. While attempting to sanitize the stock, some of the identified clean cultures showed bacterial re-emergence in the subsequent indexing. Concurrent studies undertaken with apparently clean grape cultures also showed such covert bacterial association and bacterial re-emergence, attributable partly to endophytic survival of bacteria[11,12] and partly to some bacteria that withstood the alcohol-flaming of tools. This observation has led to the isolation of two long-term, alcohol-surviving, spore-forming bacteria, identified as Gram-positive *Bacillus pumilus*[13] and Gram-negative *Brevibacillus* sp.[14]. The spent rectified spirit used during subculturing of watermelon cultures also revealed bacteria upon indexing. Since ethanol is considered to be a sterilant during tissue culture and microbial culture transfers, little attention is usually given by plant biologists and microbiologists about the possibility of horizontal spread of contaminants through this route[15]. The present study was undertaken with the objectives of isolating and identifying the alcohol-defying bacteria associated with watermelon cultures to facilitate better management of contamination, which is a serious problem for cell and tissue cultures[7,8].

Description of watermelon cultures, media used for tissue culture and bacteriological work, and the indexing procedure followed for detecting covertly associated bacteria can be found elsewhere[10]. Rectified spirit (grade 1, potable, 96% ethanol) and absolute alcohol (99.8% purity) were obtained from Mysore Sugar Company Ltd, Mandya. Additionally, analytical grade ethanol (99.9% assay) was obtained from Changshu Yangyuan Chemicals, Jiangsu, China. The spent spirit used during the subculturing of covertly contaminated watermelon cultures was first spotted (1 µl × 50) on nutrient agar (NA) followed by plating on NA (100 µl × 5). Eight apparently distinct colony types that emanated on NA were selected after 48–72 h at 25–30°C, challenged with filter-sterilized 70% ethanol for 2 h and dilution-plated. Five distinct colonies (designated as 3 × WMARB-1 to -5) were selected for further studies based on colony characteristics and microscopic observations. These isolates and two other spore-forming controls (*B. thuringiensis* and *Brevibacillus* sp.)[14] were challenged with rectified spirit, absolute or 70% ethanol for 1–7 days followed by spotting on NA. Five non-spore forming organisms, including Gram-negative *Escherichia coli* (DH5-α), *Pantoea ananatis*, *Sphingomonas* sp., *Agrobacterium tumefaciens* and one Gram-positive isolate *Microbacterium esteraromaticum* were employed as control to ascertain the general bactericidal effect of alcohol. These organisms, except *E. coli*, were isolated as endophytes from papaya shoot cultures (Thomas *et al.*, unpublished). About 51 ml fresh rectified spirit and absolute alcohol were tested for possible introduction of any bacteria through commercial supplies by plating 100 µl per NA dish (10 nos) or applying 1 ml in a plate (50 nos) and allowing the alcohol to evaporate away in a laminar hood.

Colony observations and standard staining were performed according to Cappuccino and Sherman[16]. Gram-staining was undertaken using the kit from Hi-Media Laboratories Pvt Ltd, Mumbai. Live samples were observed under phase (400 ×) and stained smears under oil (1000×) using a Zeiss Axioskop 2plus optical microscope, as described earlier[13]. Gram staining was followed up with 3% KOH mucoid thread test[17], and spore production was confirmed through malachite green staining and heat test at 80°C for 10 min[16,18].

Bacterial identification was undertaken through sequencing of 16S rDNA and homology match search of gene banks after three rounds of single-colony purifications. DNA isolation and PCR amplification using the 16S rDNA universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were undertaken as described elsewhere[13], followed by direct sequencing of PCR product using 27F primer at Macrogen Inc., Seoul, Korea (www.macrogen.com). Nucleotide sequence similarities were determined using BLAST

(version 2.2.13) search of NCBI Genbank (http://www.ncbi.nlm.nih.gov/) and were further confirmed with Seqmatch (version 2) analysis of Ribosomal Database Project (RDP-II, Release 9) of the Center for Microbial Ecology, Michigan State University (http://rdp.cme.msu.edu/seqmatch). Per cent similarity with three closest species-level sequences in the Genbank database was assessed after pairwise alignment employing ALIGN query tool (http://molbiol-tools.ca/)[19], using that part of the Genbank sequence corresponding to the region between the start and end of the sequence generated in this study. The organism was assigned to a species if the sequences were ≥99% similar with those deposited in the Genbank at the time of analysis (January 2006), according to Drancourt *et al.*[20]. Identification of 3 × WMARB-1, which showed some overlapping in 16S rRNA sequence in spite of using fresh, single colonies, was undertaken by CABI Bioscience, Egham, UK through fatty acid profiling. All media and accessories used during the study, but for the single-use 10-cm Petri dishes (Hi-Media, Mumbai), were autoclaved after affixing autoclaving indicator tapes and stringent sterile conditions were followed during isolation and subsequent handling, as described earlier[11,13].

Fresh rectified spirit and absolute alcohol upon plating did not produce any bacterial growth for over one week at 25 or 37°C, which excluded the possibility of bacterial introduction through commercial supplies. On the other hand, the spent spirit upon plating showed a bacterial titre of 0.2–0.4 × 10^4 cfu ml^{-1} the same day and similar figures on overnight storage. This indicated infection of alcohol with hardy bacterial spores during the handling of contaminated cultures and their extended survival defying the 'sterilant'. Alcohol-dipped forceps after a quick flaming just to burn-off ethanol, revealed residual bacteria in 25% of the indexed spots within 24 h and in all the remaining spots during the next 2–4 days. Generally, alcohol is expected to provide sterilization, whereas flaming is carried out primarily to evaporate ethanol[21]. Extensive flaming of contaminated tools over a Bunsen burner flame for 30–40 s did not show any residual bacteria.

The five watermelon isolates showed Gram-positive reaction and no mucoid thread formation in the KOH test, except for 3 × WMARB-2 which displayed slight tendency for Gram-negative-specific mucoid thread formation[17]. All of them were aerobic, spore-forming bacilli as revealed through phase microscopy, malachite green staining and heat-resistant spores. These as well as other spore-bearing controls showed survival in rectified spirit, absolute or 70% ethanol from overnight to over one week, as brought out through plating on NA (Figure 1). The non-spore forming checks were killed within a few minutes of challenge, indicating that spores in the former conferred the alcohol tolerance property.

Sequencing of the PCR product (approx. 1.5 kb) worked satisfactorily for all isolates, except 3 × WMARB-1 which showed overlapping sequences in spite of three repeated attempts with freshly purified single colonies. This isolate was identified through fatty-acid profiling as *B. megaterium*. Isolate 3 × WMARB-2 in BLAST search of the NCBI database showed high sequence similarity to *B. fusiformis* (99.8% identity; Table 1), followed by *B. macroides* (99.7%). This isolate was identified as *B. fusiformis* based on the sequence of 5′ hyper variable region, according to Goto *et al.*[23]. Isolate 3 × WMARB-3 was identified as *B. pumilus*, 3 × WMARB-4 as *B. subtilis* and 3 × WMARB-5 as *B. flexus*. Similar conclusions were drawn from the RDP-II sequence match search, and in repeated sequencing using fresh, single colonies from actively maintained stocks. Isolate 3 × WMARB-3 differed from grape *B. pumilus* isolate ARBG2 to the extent of 0.2% in the 632 bp 5′ sequence examined[13]. Besides, the colonies appeared morphologically different, suggesting the two as distinct isolates.

The most effective approach in *Bacillus* taxonomy is the analysis of 16S rRNA[18]. The reason for overlapping sequences in 3 × WMARB-1 is not understood. DNA admixture appeared unlikely in view of the repeated single-colony purifications attempted. The other possibility could be multiple copies of 16S rRNA gene as observed in some instances[22]. However, this warrants gene cloning and sequencing to arrive at any conclusion.

The genus *Bacillus* is a large and heterogenous collection of aerobic, rod-shaped, endospore-forming bacteria, widely distributed in the environment[18,23]. The source of above organisms in watermelon culture is obscure. The watermelon culture was initiated almost 6–7 years prior to the isolation of these organisms. Whether these organisms were introduced at culture initiation or they gained entry during the prolonged *in vitro* culturing is not definite. Several routes of entry of bacterial contaminants in plant tissue culture are suggested[8,13,24] and *Bacillus* spp. form one of the most frequently isolated bacterial contaminants[8,10].

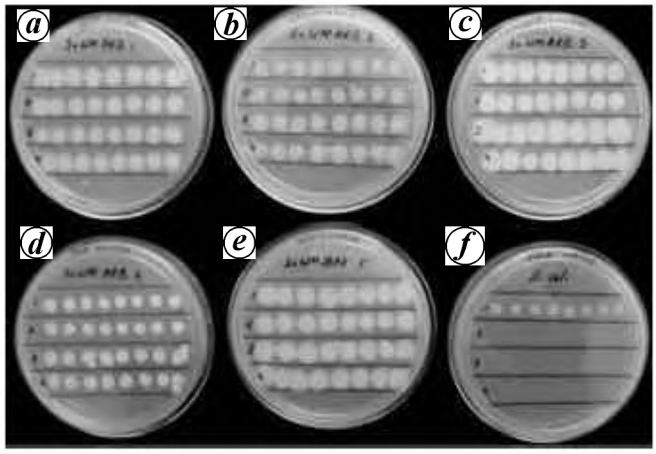

Figure 1. Spotting test of spore/colony samples (1 µl) of (***a***) *Bacillus megaterium*, (***b***) *B. fusiformis*, (***c***) *B. pumilus*, (***d***) *B. subtilis*, (***e***) *B. flexus* and (***f***) *Escherichia coli* on nutrient agar after one week of stationary incubation at 25°C in distilled water (row 1), 70% ethanol (row 2), absolute alcohol (row 3) or rectified spirit (row 4).

Table 1. Identification of alcohol-defying bacteria isolated from long-term micropropagated *in vitro* cultures of seedless watermelon based on 16S rDNA sequence similarity or fatty acid profiling

Bacterial isolate	Identification method/ 16S rDNA sequence length	Accession no. of NCBI-deposited 16S rDNA sequence	Closest species match from NCBI Genbank	
			'Strain' (accession no.)	Similarity (%)
3 × WMARB-1	Fatty acid profiling*	Not applicable	Not applicable	
3 × WMARB-2	16S rDNA/1458 bp	DQ376024	*Bacillus fusiformis* 'Z1' (AY548950)	99.9
3 × WMARB-3	16S rDNA/1450 bp	DQ376025	*Bacillus pumilus* 'GSP61' (AY505512)	100
3 × WMARB-4	16S rDNA/1452 bp	DQ376026	*Bacillus subtilis* 'WL-6' (DQ198162)	100
3 × WMARB-5	16S rDNA/1455 bp	DQ376025	*Bacillus flexus* (AB021185)	100

*Overlapping nucleotide sequences.

Alcohols are widely used as microbial disinfectants in tissue culture, microbiological and pharmaceutical establishments and in hospitals, with the impression that hazardous microbes are eliminated by the action of alcohol alone or together with flaming. This is largely true, since alcohol is effective against the vast majority of bacteria which are non-spore forming[1–5]. However, the problem may be widespread in tissue culture as *Bacillus* spores are ubiquitous[18] and one spore is enough to cause subsequent inoculum build-up[11,12]. The problem is probably going unnoticed due to the covert survival of such organisms[10,12], the general belief that alcohol is an effective sterilant and that occasionally encountered contaminants are often attributable to faulty or poor sterile practices[8]. The observations here together with reports on alcohol-tolerant *B. pumilus* and *Brevibacillus* sp. from grapes in our laboratory[13,14], substantiate this. Some earlier reports have addressed the havoc caused by alcohol-tolerant *Bacillus* in plant tissue culture[15,24–26]. It is often a practice to recycle the spirit at the laminar hood by successive workers. Once contaminated, it may serve as the source of inoculum. Bacteria which survive without any apparent hazardous effects in the cultures of one plant may turn overt and detrimental in other cultures, as observed with grape isolate of *B. pumilus* in papaya and in watermelon cultures[13]. It is also a matter of concern that the dreaded anthrax pathogen, *B. anthracis* belongs to the same genus as the above organisms[18], and general alcohol wipes and sprays will not be effective in eliminating the hardy spores. Further investigations on the probable source of these organisms in watermelon cultures, extent of spore longevity in varying concentrations of ethanol and ways to circumvent the menace are underway.

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Rice variety Dhanrasi, an example of improving yield potential and disease resistance by introgressing gene(s) from wild species (*Oryza rufipogon*)

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Rice variety Dhanrasi (C 11-A-41) was developed by introgressing gene(s) for yield enhancement, resistance to blast and moderate resistance to bacterial blight and rice tungro disease from wild species (*Oryza rufipogon*). The *O. rufipogon* accession resistant to blast, bacterial blight and rice tungro disease was crossed with high-yielding line B 32-Sel-4. The F_1 (B 32-Sel-4 × *O. rufipogon*) was crossed with another high-yielding line B 127. Two cycles of selective intermating were followed in the F_2 generation and thereafter pedigree method of selection was continued. In the F_5 generation 32 lines were evaluated with the *indica* parents for yield, and also screened for blast and bacterial blight resistance. Eight lines were superior in yield to both the *indica* parents by 10.2–21.4%, indicating introgression of yield-enhancing gene(s) from *O. rufipogon*. Culture C 11-A-41 yielded the highest (6.48 t/ha) with yield superiority of 38.2 and 21.4% over its parents B 32-Sel-4 and B 127 respectively. The present results indicated that the gene(s) for yield enhancement, resistance to blast and moderate resistance to bacterial blight and rice tungro disease in C 11-A-41 were introgressed from *O. rufipogon*. Considering its yield superiority and multiple resistance to diseases, the culture (C 11-A-41) was released as variety Dhanrasi for cultivation in Andhra Pradesh, Tamil Nadu, Karnataka and Maharashtra in 2002.

Keywords: Biotic stress resistance, introgression, *O. rufipogon*, variety Dhanrasi, yield-enhancing gene(s).

RICE productivity in irrigated and rainfed shallow lowland areas in India has remained almost stagnant for a long time after the release of varieties Salivahana and Pranava in 1988. Since then several breeding lines have been evaluated in these ecosystems under the All India Coordinated Rice Improvement Project (AICRIP) with Salivahana and Pranava as the national checks, but none could qualify for release in irrigated shallow lowlands in the southern region[1]. This showed that the yield improvement *per se* in the lines bred for these ecosystems specially in irrigated shallow lowland remain limited, though there has been considerable improvement in quality and pest resistance in varieties released at the state-level. The possible reason for low genetic gain in yield may be the narrow genetic base due to the fact that a small core of adapted germplasm has been used repeatedly in breeding programmes.

Wild species of *Oryza* are important reservoirs of genes for agronomically important traits such as resistance to biotic and abiotic stresses, improved quality characteristics and yield[2]. A number of major genes showing resistance to bacterial blight (*Xa21*, *Xa23*), blast (*Pi9*), rice tungro disease, brown plant hopper (*Bph10*, *bph12*, *Bph13*, *Bph14*, *Bph15* and *Bph18*) and grassy stunt virus have been introgressed from different wild species using the backcross breeding method[2]. Recent studies using molecular markers have demonstrated that wild relatives are also important sources of useful alleles for complex traits such as yield in crops like tomato[3] and rice[4]. Though several yield-enhancing quantitative trait loci (QTLs) have been identified from different species of wild rice such as *Oryza rufipogon*[5–7], *O. nivara*[8] and *O. glumaepatula*[9], no variety has been so far released having known yield-enhancing genes/QTLs introgressed from wild rice. Combining the favourable yield-enhancing genes/QTLs located on different chromosomes in one background following repeated backcross breeding method is a difficult

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