Moreno-Vázquez, S., Larrañaga, N., Uberhuaga, E. C., Braga, E. J. B., & Pérez-Ruiz, C. (2014). Bacterial contamination of *in vitro* plant cultures: confounding effects on somaclonal variation and detection of contamination in plant tissues. *Plant Cell, Tissue and Organ Culture*, *119*(3), 533–541. doi: 10.1007/s11240-014-0553-x

Bacterial contamination of *in vitro* plant cultures: confounding effects on somaclonal variation and detection of contamination in plant tissues

Moreno-Vázquez Santiago [1*]; Larrañaga Nerea [1]; Uberhuaga Elizabeth C [2]; Braga Eugenia Jacira Bolacel [1,3]; Pérez-Ruíz César [1]

[1] Departamento de Biología Vegetal, E.T.S. Ingenieros Agrónomos, Universidad Politécnica de Madrid, Ciudad Universitaria, 28040 Madrid, Spain; [2] Laboratorio de cultivo *in vitro* del Ayuntamiento de Madrid, Autovía M30 p.km 22.8, 28040 Madrid, Spain; [3] Departamento de Botânica - Instituto de Biologia, Universidade Federal de Pelotas, Campus Universitário, 96010900 Pelotas – RS, Brazil. (* author for correspondence, e-mail santiago.moreno@upm.es, telephone 34-913365661, fax 913365656)

Abstract

Bacterial contamination represents a serious problem for plant tissue culture research and applications. Bacterial interference with normal plant physiology and morphology can generate misleading conclusions if the presence of bacteria is ignored. Bacterial contaminants in *in vitro* plant culture are typically detected by direct observation; thus, it is assumed that cultures without visible symptoms are bacteria free. Here, we demonstrate that contaminating *Bacillus* DNA in plant DNA solutions from asymptomatic plants can interfere with the analysis of somaclonal variation in chrysanthemum. We studied somaclonal variation in chrysanthemum using short semi-specific PCR primers based on conserved motifs in NBS-LRR disease resistance genes and in mobile elements. Instead of true somaclonal variation we found three polymorphic bands derived from contaminant bacterial DNA in plant extracts. Although the detection of asymptomatic bacteria in *in vitro* plant cultures is a major issue, we found that it has not been adequately addressed to date, particularly for studies on somaclonal variation. We reviewed the most commonly cited contaminant bacteria in *in vitro* plant culture and designed specific 16S rRNA gene-based PCR primers for the main genera causing contamination (*Bacillus, Pseudomonas, Staphylococcus, Lactobacillus, Erwinia/Enterobacter and Xanthomonas*). Using a panel of pure bacterial

DNAs, artificial mixes of bacterial/plant DNAs, and *in vitro* plant cultures with and without visible contamination we demonstrated that our primers are in most instances both reliable and sensitive, and appropriate for the identification and tracking of the most frequent bacterial contaminats in plant *in vitro* cultures. Implications of bacterial identification to molecular analysis of somaclonal variation and plant culture decontamination are discussed.

Key words

Somaclonal variation, 16S rRNA, *Bacillus, Staphylococcus, Pseudomonas, Lactobacillus, Erwinia, Enterobacter*, *Xanthomonas*.

Key Message

Bacteria from the genus *Bacillus* are demonstrated to interfere with and confound somaclonal variation studies in plants. Genus-specific PCR markers for the main bacterial genera causing contamination in *in vitro* plant cultures (*Bacillus, Pseudomonas, Staphylococcus, Lactobacillus, Erwinia, Enterobacter* and *Xanthomonas*) are proposed and evaluated.

INTRODUCTION

Contaminant organisms in *in vitro* plant cultures include viruses, bacteria, yeasts, fungi, mites and thrips. Bacterial contamination has been widely reported because of the serious problems it can cause (Leifert et al. 1991; Reed and Tanprasert 1995). Most bacterial contamination of *in vitro* plant cultures is caused by the inefficient sterilization of explants, culture vessels, instruments or media or occurs when handling the plant material (Leifert et al. 1991). Contaminant bacteria might alter normal plant physiology and morphology, interfere with the reproducibility of culture protocols, cause serious problems for *in vitro* gene banks, prevent the safe exchange of germplasm and result in misleading conclusions in research studies if the contamination is ignored (Thomas 2004).

Although bacterial contamination can often be visually detected, in some instances, bacteria do not produce obviously detectable symptoms but still propagate together with the plant material. These bacteria are “latent”, “internal”, “endophytic” or “endogenous” bacteria (Leifert et al. 1991). Regardless of the origin of contaminant bacteria (internal or external), there is a need for simple, reliable, sensitive and specific detection methods for bacteria in plant tissue cultures. Traditional detection techniques involve bacteria isolation and cultivation. However, molecular detection based on direct DNA analysis makes these time-consuming steps unnecessary and is sensitive enough for most groups of bacteria (Lopez et al. 2009). Molecular detection by PCR is generally based on the analysis of three types of sequences: pathogenesis-related genes, plasmid genes and ribosomal operons (Garcia-Martinez et al. 1999; Daffonchio et al. 2000; Lopez et al. 2009). Ribosomal operons include the relatively well-conserved 16S, 23S and 5S genes and interspersed relatively variable regions known as internal transcribed spacers (ITS). The 16S rDNA gene, which includes conserved and variable regions, is the most useful fragment for bacterial identification at the genus level (Huys et al. 2008). PCR analysis of 16S rDNA fragments for purposes of bacterial identification has been widely used and is a well-established methodology for environmental, water, clinical and food samples (Grahn et al. 2003). Nonetheless, there is a lack of research on the use of 16S rDNA-based PCR for the detection of bacterial contaminants in *in vitro* plant cultures.

Here, we first report how bacteria from the genus *Bacillus* interfered with a study on somaclonal variation in chrysanthemum. Second, we review the genera of bacteria most

frequently cited as contaminants in *in vitro* cultures. Based on this review, we designed and evaluated genus-specific 16S rDNA-based PCR primers for the detection of contaminant bacterial DNA in plant DNA extracts from symptomatic and asymptomatic *in vitro* plant cultures.

MATERIAL AND METHODS

In vitro plant cultures

The analysis of somaclonal variation was performed on three chrysanthemum (*Dendranthema grandiflora*) tissue culture lines. These lines were initiated using two individuals from the cultivar "Refocus" (RF16 and RF24) and one from the cultivar "Red Reagan" (RR11) as mother plants. Nine generations per line were examined. One generation corresponded to a subculture step. Each culture vessel for a given generation was subcultured into two new culture vessels. Each line was derived from a mother plant through an initial step of meristem culture (MS+0.1 mg/l NAA+0.1 mg/l BAP; for abbreviations see Table 1) and with subsequent monthly shoot micropropagation up to 9 generations (MS+0.1 mg/l NAA+0.2 mg/l BAP). Each culture was subcultured into two new culture vessels. Each subculture step was considered a new generation.

The plant cultures for testing genus-specific primers are described in Table 1. All the cultures were derived from routine work at a micropropagation laboratory. Except for control cultures A and B, all of the remaining cultures were selected for apparent symptoms of bacterial contamination in the growth medium. However, none of the cultures analyzed showed visual symptoms of bacterial contamination associated with plant tissues. Glass culture vessels with plastic covers (LABASSJAR38, Lab Associates B.V.) were generally used. In two instances (cultures B and G), plastic containers (ECO2BOX green filter, Duchefa Biochemie B.V.) were used instead. A solid MS-based plant growth medium with species-dependent additives was used (Table 1).

PCR reactions

Each PCR was performed in a volume of 20 µl containing 2 mM $MgCl_2$, 75 mM Tris-HCl (pH 9), 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 200 µM dNTP, 0.5 µM oligonucleotide primer (each), 0.7 units/reaction Taq polymerase (Biotools ref. 10.042) and 25 ng template DNA. The thermocycler (Eppendorf Mastercycler) was programmed as

follows: initial denaturing at 94 ºC for 4 min, followed by 35 cycles of, denaturation at 94 ºC for 40 s, annealing at 45 to 70 ºC for 40 s and extension at 72 ºC for 1 min to 1 min 15 s. A final extension step of 72 ºC for 7 min was included. The annealing temperature and extension time varied depending on the primer pair used. For the studies on somaclonal variation, the annealing temperature ranged from 45 ºC to 50 ºC;for bacterial detection, the range was from 65 ºC to 70 ºC. Amplicons were separated by electrophoresis through 2% agarose gels and visualized with ethidium bromide staining.

Primer selection and design

For the detection of somaclonal variants in chrysanthemum, semi-specific short PCR primers (Online Resource 1) were designed based on two conserved motifs within the NB-ARC domain of NB-LRR resistance genes (N primers) and on multiple conserved motifs within transposable elements (M primers). Our goal was to evaluate the extent to which the stress produced by *in vitro* culture might introduce variation associated with these two types of plastic genome elements (Schmidt and Anderson 2006; Friedman and Baker 2007; Jurka et al. 2007). The N primers were targeted to either the N-terminal P-loop (primers NP) or the C-terminal hydrophobic motif (primers NH). The M primers targeted high-copy number transposable elements that preferentially insert into gene-coding regions (Casacuberta and Santiago 2003). The design of the primers was based on the consensus sequences obtained after annealing NB-ARC sequences from other members of the Asteraceae family (*Helianthus, Lactuca*) and from dicot species that are not members of this family. The length of most M and N primers was 15-16 bp. Primers N and M were used either alone or in combination as follows: N with M, NP with NH or NP with NP. We combined only primers with similar melting temperatures and not forming primer-dimer structures.

For the detection of bacterial contaminant DNA in plant DNA extracts, we attempted to use both universal primers from the literature (Louws et al. 1999) and genus-specific primers for 16S rDNA designed in our laboratory. To design the genus-specific primers, we first reviewed the literature to identify the most cited genera of contaminant bacteria in *in vitro* plant cultures. Then, we downloaded the 16S rDNA sequences encoded by these genera from *Ribosomal Database Project* Release 10 Update 25 (Cole et al. 2009). We also downloaded chloroplastic and mitochondrial 16S rDNA sequences from

diverse plant species (*Arabidopsis thaliana, Solanum nigrum, Populus alba, Oryza sativa, Tritium aestivum* and *Zea mays*). All of the downloaded 16S rDNA sequences were aligned using MULTALIN (Corpet 1988). Using Primer 3 version 4.0 (Rozen and Skaletsky 2000), pairs of genus-specific primers were designed while attempting to place the 3' end of each primer in a genus-specific region of the template and with melting temperatures of approximately 60 ºC. We also ensured that each designed primer had at least three mismatches to the homologous chloroplast and mitochondrial sequences to prevent amplification from these templates.

For the identification of bacteria growing on the surface of solid growth media in *in vitro* cultures, direct PCR (Hiraishi 1992) was performed with the universal prokaryote primers FPL06 and RDR245 (Greisen et al. 1994).

Template DNA

The analysis of somaclonal variation was performed on the three chrysanthemum tissue culture lines mentioned above. For each line, in each generation, we sampled tissue from only a single culture vessel. The sampled vessel of a given generation was always derived from a culture vessel that was sampled in the previous generation. DNA was extracted according to Weising et al. (2005) from 100 mg of leaf tissue in cultures lacking apparent symptoms of bacterial or fungal contamination. In general, two DNA extractions were performed for each line and generation, both from the same culture vessel.

The specificity of the PCR primers for bacterial detection was verified using a panel of pure bacterial DNA solutions hereafter referred to as 'control bacterial DNA.' The panel consisted of the seven following bacteria strains representing the genera most cited as contaminants in *in vitro* plant cultures: *Lactobacillus plantarum* (CECT220); *Pseudomonas fluorescens* (CECT378); *Bacillus subtilis* subsp. *spizizenii* (CECT356); *Staphylococcus saprophyticus* subsp. *saprophyticus* (CECT235) supplied by The Spanish Type Culture Collection at the Universidad de Valencia, Spain; *Pseudomonas syringae* (DC3000) and *Dickeya dadantii* (3937, ex *Erwinia chrysanthemi*) supplied by Dr. Emilia López-Solanilla from the Centre for Plant Biotechnology and Genomics UPM, Madrid, Spain; *Xanthomonas campestris* supplied by Dr. Jaime Cubero from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria. Bacterial DNA

was extracted at the Center for Plant Biotechnology and Genomics using the "Easy-DNA kit for genomic isolation" (Invitrogen). Serial dilutions of the different bacterial control DNA solutions were used to evaluate the sensitivity of the genus-specific primers. Two sets of serial dilutions (5 to 5 x 10^{-5} ng/µl) of bacterial DNA were prepared. In the first set, distilled water was used as the solvent. In the second set, the solvent was a 5 ng/µl water solution of plant DNA extracted from sample A1 (Table 1).

Genus-specific PCR primers for bacterial detection were also tested using templates consisting of DNA extracts from plant tissue derived from *in vitro* cultures with visible symptoms of bacterial contamination on the surface of the growth medium (C to K in Table 1 and Figure 1). Control cultures with no apparent signs of bacterial contamination were also included (A and B in Table 1). The plant DNA was extracted from 100 mg of leaf tissue according to Weising et al. (2005). Whenever possible, two samples of leaf tissue were taken from each culture: sample one consisted of leaves located relatively close to the growth medium but not touching it (sub-index 1 in Table 1), and sample two consisted of leaves relatively distant from the growth medium (sub-index 2).

To identify bacteria on the growth medium of *in vitro* cultures, we performed PCR with the universal primers described above using template bacteria cells without previous DNA isolation (Hiraishi 1992). To obtain separate colonies, the bacteria were obtained from the gel surface and streaked onto both MS and King'B solid media (Murashige and Skoog 1962; Schaad 1988) and grown at 29 ºC for at least 5 days.

Sequencing

PCR amplifications obtained with bacteria universal and also amplifications from genus-specific primers were sequenced to gain insight into bacterial identification. Prior to sequencing, the PCR reactions were purified using "Speedtools PCR Clean-Up*"* (Biotools). Sequencing was performed by Secugen S.L. (Madrid, Spain) using an Applied Biosystem capillary sequencer 3730 DNA Analyzer.

RESULTS AND DISCUSSION

The analysis of somaclonal variation in chrysanthemum using semi-specific PCR primers (Online resource 1) resulted in three polymorphic and reproducible bands from

line RR 11 (results not shown): a 2200-bp band from the 17-mer primer HDNT6, a 1993-bp band from the 12-mer primer SINE1 and a 1300-bp band from the 17-mer primer PHNT2. All of these bands were present in the seventh and ninth generation but absent in other cases and showed high homology to *Bacillus* genes: methionyl-tRNA ligase, UGMP family protein and CoA-binding domain protein, respectively. We hypothesized that these three bands did not represent actual somaclonal variation but instead were caused by contaminant bacteria in chrysanthemum cultures that lacked visible symptoms. To confirm this hypothesis, we performed PCR using *Bacillus*-specific primers (Table 2) and the different RR11 chrysanthemum template DNAs. As expected, only DNA templates from the seventh and ninth generations produced amplification (results not shown). Most likely, *Bacillus* cells contaminated line RR11 for the first time during the seventh generation and were transmitted to the ensuing generations from this initial inoculum. Interestingly, the tissue sample from the eighth generation used for DNA extraction did not appear to contain bacteria, even though the sample used to produce the ninth generation very likely did contain bacteria. In this case, it is possible that the contaminant bacteria did not spread through the entire body of the contaminated eighth generation plant. In summary, we confirmed our initial hypothesis and demonstrated that the presence of asymptomatic *Bacillus* bacteria can interfere with the analysis of plant somaclonal variation when using semi-specific PCR. We conclude that in somaclonal variation studies performed with non-specific (RAPDs, ISSR, AFLP) or semi-specific PCR, it is always advisable to determine the sequence of any candidate polymorphic DNA to discount interference from contaminant bacteria.

We reviewed 24 case studies on the use of non-specific PCR for the detection of somaclonal variants in *in vitro* plant cultures (Online Resource 2) and found that only six included an identification of the polymorphic bands via sequencing. Only one of the 18 remaining studies addressed possible confounding interference from bacteria, fungi or other biological contaminants. The analysis of somaclonal variation on sweet potato by RAPDs reported by Villordon and Labonte (1995) is the exception. In this work, although the polymorphic bands were not sequenced, the authors proposed a procedure to minimize contamination from *Fusarium lateritium* or virus-like organisms that might serve as alternative templates for amplification and confound data analysis. Concerning bacterial contaminants specifically, Thomas et al. (2008) investigated whether certain bacteria could play a role in somaclonal variation. To our knowledge, the issue has not

been further addressed to date. Based on our literature review, we conclude that most studies on somaclonal variation using non-specific PCR published thus far were unable to ascertain the true origin of the variation found.

Another way to minimize such confounding effects would be to detect contaminant bacteria prior to the analysis of somaclonal variation. However, beyond purification using standard bacteriological methods, culture indexing systems and morphological and biochemical tests, there are no reports in the literature on molecular methods to specifically detect bacteria contaminants in *in vitro* plant cultures. Thus, we attempted to fill this gap by designing reliable PCR primers for the detection of contaminant bacteria in *in vitro* plant cultures, as described above.

First, we sought to detect contaminant bacteria in *in vitro* plant cultures using universal bacteria primers specific for 16S rDNA (see Material and Methods). As test samples, we used several cultures both with and without visible contamination on the growth medium but always without plant symptoms (A to K in Table 1). Using these primers, all samples produced amplification products, even plant DNA extracted from cultures without any symptoms of bacterial contamination (results not shown). This result might be due to the amplification of contaminating bacterial 16S rDNA fragments in the PCR reagents (Grahn et al. 2003; Chang et al. 2011). Consequently, to detect bacterial contamination in plant DNA extracts, we designed better primers specific for the bacterial genera most commonly found in *in vitro* plant cultures.

To identify these genera, we searched 100 citations in the literature on bacterial contaminants in *in vitro* plant cultures (Figure 2). *Bacillus* and *Pseudomonas,* each accounted for almost 20% of the citations. The elevated incidence of *Bacillus* contamination could be due to poor medium or instrument sterilization because their spores (endospores) can in some instances survive standard autoclave conditions (Leifert and Woodward 1998). The reason why *Pseudomonas* is also frequently found as a contaminant in *in vitro* plant cultures might be due to the ubiquitous presence of this genus on plant surfaces (Tamprasert and Reed 1997). *Xanthomonas*, *Micrococcus*, *Enterobacter*, *Staphylococcus*, *Erwinia* and *Lactobacillus* each accounted for approximately 5 to 8% of the citations.

With the exception of *Micrococcus*, we designed genus-specific PCR-primers for these genera, as described in the Materials and Methods section. Details on the primer features and amplification conditions are summarized in Table 2. Primer specificity was verified using bacterial DNA controls as templates (lines 3 to 8 plus line X and X^{-1} in Figure 3). The designed primers were found to be highly specific in all cases. The primers for *Staphylococcus* (Figure 3c) and *Lactobacillus* (Figure 3d) also amplified relatively faint bands of the expected size when template DNA from other species was used. These relatively much less intense bands can be readily distinguished from the strongest specific bands and consequently do not affect the applicability of the primers. To test the PCR sensitivity, serial dilutions of bacterial control DNA using distilled water and plant DNA solutions as the diluent, were prepared as detailed in Figure 4. The PCR primers for *Bacillus*, *Pseudomonas, Staphylococcus* and *Xanthomonas* could detect bacterial DNA at concentrations as low as 5.10^{-5} ng/µl (Figure 4a, 4b, 4c and 4f). The primers for *Lactobacillus* were the least sensitive, requiring at least 5 ng/µl of bacterial DNA for detection (Figure 4d), and the primers for *Erwinia/Enterobacter* had an intermediate sensitivity (Figure 4e). No differences in sensitivity were found in any case when comparing the specificity of primers resulting from the two types of serial dilutions. However, when using control plant DNA (A1 in Table 1, lane 9 in Figure 4) specific primers for *Bacillus* and *Staphylococcus* produced bands of the expected size but very faint compared to bands obtained using serial dilutions as templates. We assume that the primer design ensures that neither cpDNA or mitochondrial DNA extracted from plant sample A1, nor *E. coli* DNA introduced with the DNA polymerase would serve as templates to produce amplifications with any of the bacterial primers. Then, the easiest explanation for these bands could be that they were caused by a very small amount of an asymptomatic bacteria (closer to *Bacillus* and *Staphylococcus* than to any of the other bacteria considered in Table 2) present in the plant sample A1. Finally, is worth to mention that the primers for *Bacillus*, *Pseudomonas*, *Staphylococcus* and *Xanthomonas* could be multiplexed in the same PCR reaction because they all have a similar annealing temperature, with no primer-dimer formation expected, and the size difference between the PCR products is larger than 80 bp.

The developed genus-specific markers were also used to detect contaminating bacterial DNA in 11 *in vitro* cultures derived from standard manipulation in a micropropagation laboratory (cultures A to K in Table 1). A total of 17 plant DNA extracts (lines 9 to 25

in Figure 3) were analyzed. Cultures A and B, without visible symptoms of bacterial contamination, were considered the control cultures. Cultures C to K showed visual symptoms of bacterial presence only on the growth medium surface. For each of cultures C to K, we utilized two plant tissue samples whenever possible: one sample close to the gel surface (subscript 1), and one sample relatively far from the gel surface (subscript 2). The PCR results revealed possible contamination with *Pseudomonas* in sample K1 (Figure 3b, lane 25), *Erwinia/, Enterobacter* in sample J1 (Figure 3e, lane 24) and *Xanthomonas* in samples G2 and J1 (Figure 3f, arrows in lanes 20 and 24). The remaining 14 plant DNA extracts did not show significant evidence of contamination. To confirm these results, the PCR products were sequenced (Online Resource 3), and BLAST was used to identify homologous sequences in GenBank (Release 184.0). As expected, the amplicon obtained using *Pseudomonas*-specific primers with template K1 showed sequence homology to *Pseudomonas* 16S rRNA, the amplicon using *Erwinia/Enterobacter* primers with J1 showed homology to *Enterobacter* 16S rRNA, and the amplicon using *Xanthomonas* primers with template J1 showed homology with *Rhodanobacter* (Xanthomonadaceae). The sequence of the amplicon from template G2 using *Xanthomonas*-specific primers could not be determined. In summary, the analyzed sequences validated the results for the presence of bacteria based on our genus-specific primers in all cases.

To gain insight into the different species of bacteria most commonly found on the gel surface of *in vitro* plant cultures under normal micropropagation procedures, we studied the gel surface in cultures C to K (Table 1, Online Resource 3) and in 14 additional cultures (L to Y, Online Resource 3), all derived from normal activity in a micropropagation laboratory. As detailed in Material and Methods, the bacteria were obtained from the gel surface and streaked onto KB and MS plates. Separate colonies were subjected to direct PCR with universal primers targeting bacterial 16S rDNA. For colony identification, the PCR products were sequenced, and BLAST was used to identify homologous sequences in GenBank. The sequences are shown in Online Resource 3. For cultures C to K, we were able to identify the contaminating bacteria only for cultures E, G, H, I and K. The bacteria from the other cultures (C, D, F and J) could not be grown in KB or in MS media and therefore could not be identified. For culture K, the contaminant bacteria (*Pseudomonas*) found on the gel (Online Resource 3) coincides with the contaminant bacteria on the corresponding plant tissue (K_1 in

Table 1). For culture G, *E. coli* was found on the gel (Online Resource 3), whereas only *Xanthomonas*-specific primers were able to amplify DNA using plant tissue (G_2 in Table 1). To test whether *E. coli* DNA could be amplified with *Xanthomonas* or other genus-specific primers in Table 1, several 16S rDNA *E.coli* sequences were downloaded from the Ribosomal Database Project and aligned to these primers. The alignments indicated that only *Enterobacter/Erwinia* specific primers would be able to amplify *E.coli* DNA. This is not an unexpected result because *E. coli* belongs to the Enterobacteriaceae family. Therefore, the plant tissue DNA extracted from culture G would contain *Xanthomonas* DNA and would be *E. coli* free. For the 14 additional cultures, we identified bacteria belonging to genera *Pseudomonas* (sample L), *Rhodanobacter* (sample M), *Microbacterium* (=*Brevibacterium)* (sample N), *Curtobacterium/Bacillus* (samples O, P, Q, S and T), *Paenibacillus* (samples R and W), *Staphylococcus* (sample X), and *E. coli* (in samples U and V). All these bacteria except *E. coli* have been cited in the literature as important contaminants of in vitro plant cultures. Therefore, except for *E. coli*, our study on the bacteria most commonly found on the gel surface of in vitro plant cultures under normal micropropagation procedures confirm expectations based on the literature.

Overall, our findings suggest that, even in the absence of visible symptoms of bacterial contamination, *in vitro* cultures should be analyzed to rule out bacterial contamination before carrying out somaclonal or other studies in which bacteria might confound the results. The set of genus-specific primers proposed here could be a valuable resource for discovering and tracking contamination in *in vitro* plant cultures. The identification of the bacteria in *in vitro* plant cultures has an additional advantage: when using antibiotics for decontamination, the selection of the most efficient compound can be refined using this information. Choosing the appropriate antibiotic will improve decontamination and reduce the secondary effects of their use on plants.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Emilia López Solanilla (Centre for Plant Biotechnology and Genomics, *Universidad Politécnica de Madrid* (UPM)–*Instituto Nacional de Investigación Agraria y Alimentaria* (INIA)) for providing DNA extracts from control bacteria, for carefully reviewing the manuscript and for her valuable suggestions. Additionally, we would like to thank Dra. M. Carmen Martín (Dept. Plant

Biology UPM) for providing the plant tissue used to analyze somaclonal variation and also to Dr. Jaime Cubero (INIA) for supplying control *Xanthomonas campestris*. We are also grateful to Manuel Chimeno (*Laboratorio de cultivo in vitro del Exmo. Ayuntamiento de Madrid*) for providing some of the in vitro plant cultures analyzed. The author BEJB was supported by a scholarship from the Fundación Carolina (*Agencia Española de Cooperación Internacional*). This research work was supported by a project grant (DGI AGL2004-01929/AGR) from the CICYT (Spain), and it also benefited from research agreement (FPA040000BIO100) between UPM and the *Exmo. Ayuntamiento de Madrid* (*Área de Gobierno de Medio Ambiente y Servicios a la Ciudad*).

REFERENCES

Casacuberta JM, Santiago N (2003) Plant LTR-retrotransposons and MITEs: control of transposition and impact on the evolution of plant genes and genomes. Gene 311:1–11.

Chang S-S, Hsu H-L, Cheng J-C, Tseng C-P (2011) An efficient strategy for broad-range detection of low abundance bacteria without DNA decontamination of PCR reagents. PLoS One 6:e20303. doi: 10.1371/journal.pone.0020303

Cole JR, Wang Q, Cardenas E, et al. (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res 37:D141–D145.

Corpet F (1988) Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res 16:10881–10890.

Daffonchio D, Cherif A, Borin S (2000) Homoduplex and heteroduplex polymorphisms of the amplified ribosomal 16S-23S internal transcribed spacers describe genetic relationships in the "*Bacillus cereus* group". Appl Environ Microbiol 66:5460–5468.

Friedman AR, Baker BJ (2007) The evolution of resistance genes in multi-protein plant resistance systems. Curr Opin Genet Dev 17:493–499.

Garcia-Martinez J, Acinas SG, Anton AI, Rodriguez-Valera F (1999) Use of the 16S-23S ribosomal genes spacer region in studies of prokaryotic diversity. J Microbiol Methods 36:55–64.

Grahn N, Olofsson M, Ellnebo-Svedlund K, et al. (2003) Identification of mixed bacterial DNA contamination in broad-range PCR amplification of 16S rDNA V1 and V3 variable regions by pyrosequencing of cloned amplicons. FEMS Microbiol Lett 219:87–91.

Greisen K, Loeffelholz M, Purohit a, Leong D (1994) PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. J Clin Microbiol 32:335–51.

Hiraishi A (1992) Direct automated sequencing of 16s rDNA amplified by polymerase chain-reaction from bacterial cultures without DNA Purification. Lett Appl Microbiol 15:210–213.

Huys G, Vanhoutte T, Joossens M, et al. (2008) Coamplification of eukaryotic DNA with 16S rRNA gene-based PCR primers: possible consequences for population fingerprinting of complex microbial communities. Curr Microbiol 56:553–7.

Jurka J, Kapitonov V V, Kohany O, Jurka M V (2007) Repetitive sequences in complex genomes: structure and evolution. Annu Rev Hum Genet 8:241–259.

Leifert C, Ritchie JY, Waites WM (1991) Contaminants of plant-tissue and cell-cultures. World J Microbiol Biotechnol 7:452–469. Leifert C, Woodward S (1998) Laboratory contamination management: the requirement for microbiological quality assurance. Plant Cell Tiss Org 52: 83-88.

Lopez MM, Llop P, Olmos A, et al. (2009) Are molecular tools solving the challenges posed by detection of plant pathogenic bacteria and viruses? Curr Issues Mol Biol 11:13–45.

Louws FJ, Rademaker JLW, de Bruijn FJ (1999) The three Ds of PCR-based genomic analysis of phytobacteria: Diversity, detection, and disease diagnosis. Annu Rev Phytopathol 37:81–125.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497.

Reed BM, Tanprasert P (1995) Detection and control of bacterial contaminants of plant tissue cultures . A review of recent literature. Plant tissue cult biotechnol 1: 137–142.

Rozen S, Skaletsky HJ (1999) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics methods and protocols. Humana Press, Totowa, NJ, pp 365–386.

Schaad NW, Jones JB, Chun W, (Eds.) (2001) Laboratory guide for identification of plant pathogenic bacteria. Vol 373. APS press, Minessota.

Schmidt AL, Anderson LM (2006) Repetitive DNA elements as mediators of genomic change in response to environmental cues. Biol Rev 81:531–543.

Thomas P (2004) A three-step screening procedure for detection of covert and endophytic bacteria in plant tissue cultures. Curr Sci -Bangalore- 87:67–72.

Thomas P, Swarna GK, Patil P, Rawal RD (2008) Ubiquitous presence of normally non-culturable endophytic bacteria in field shoot-tips of banana and their gradual

435 activation to quiescent cultivable form in tissue cultures. Plant Cell Tissue Organ
436 Cult 93:39–54.

437 Tramprasert P, Reed BM (1997) Detection and identification of bacterial contaminants
438 from strawberry runner explants. In Vitro Cell Dev Biol--Plant 33:221-226

439 Villordon AQ, LaBonte DR (1995) Variation in randomly amplified DNA markers and
440 storage root yield in 'Jewel' sweetpotato clones. J Am Soc Hortic Sci 120:734–740.

441 Weising K, Nybom H, Wolff K, Kahl G (2005) DNA fingerprinting in plants:
442 principles, methods, and applications. CRC Press, Boca Raton

443

Fig. 1 Analyzed i*n vitro* cultures with bacterial contamination on the surface of the growth media analyzed. (a) *Rosa* sp., code C in Table 1, (b) *Pelargonium* sp., D in Table 1, (c) *Spathiphyllum* sp., E in Table 1, (d) *Dendrathema* sp., F in Table 1, (e) *Ficus elastica*, G in Table 1, (f) *Ficus elastica*, H in Table 1, (g) *Dahlia* sp., I in Table 1, (h) *Spathiphyllum* sp., J in Table 1

Fig. 2 Number of times each bacterial genus is cited in the literature as a contaminant in plant *in vitro* cultures. A total of one hundred citations were considered

Fig. 3 PCR primer specificity with a panel of pure bacterial DNA and performance using plant DNA extracts. Photographs are after agarose gel electrophoresis of 16S rRNA fragments amplified from different template samples with primers specific for: (a) *Bacillus*, (b) *Pseudomonas*, (c) *Staphylococcus*, (d) *Lactobacillus*, (e) *Erwinia/Enterobacter* and (f) *Xanthomonas*. Lanes 1 and 26 contain a 1 kb ladder, lane 2 contains control water. Lanes 3 to 8 plus lanes X and X^{-1} show amplifications using a panel of pure bacterial DNA as templates: (3) *Lactobacillus plantarum*, (4) *Pseudomonas fluorescens*, (5) *Bacillus subtilis* subs. *spizizenii*, (6) *Staphylococcus saprophyticus* subs. *saprophyticus*, (7) *Pseudomonas syringae*, (8) *Dickeya dadantii*, (X and X-1) *Xanthomonas campestris*. Lanes 9 and 10 show amplifications using plant DNA extracts from *in vitro* cultures without any symptoms of bacterial contamination. The performance of primers with plant DNA extracts from in vitro cultures with visible symptoms of bacterial contamination on the growth medium (Table 1) is shown in lanes 11 to 25: (11) C1, (12) C2, (13) D1, (14) D2, (15) E1, (16) E2, (17) F1, (18) F2, (19) G1, (20) G2, (21) H1, (22) I1, (23) I2, (24) J1, (25) K1

Fig. 4 Sensitivity of PCR primers for the detection of contaminant bacteria. Agarose gel electrophoresis of 16S rRNA PCR-amplified fragments: (a) *Bacillus*-specific primers with *Bacillus subtilis* subsp. *Spizizenii* templates, (b) *Pseudomonas* primers with *Pseudomonas syringae* templates, (c) *Staphylococcus* primers with *Staphylococcus saprophyticus* subsp. *Saprophyticus* templates, (d) *Lactobacillus* primers with *Lactobacillus plantarum* templates, (e) *Erwinia/Enterobacter* primers with *Dickeya dadantii* (ex *Erwinia chrysanthemi*) templates and (f) *Xanthomonas* primers with *Xanthomonas campestris*templates. Lane 1 contains a 1kb ladder; lane 2 control water; lanes 3 to 8 correspond to serial dilutions (5 ng/µl, 0,5 ng/µl, 0,05 ng/µl, 0,005 ng/µl, 0,0005 ng/µl, 0,00005 ng/µl) of bacterial DNA using water as the solvent; lane 9 control plant DNA (sample A1 in Table 1); lanes 10 to 15 correspond to serial dilutions of bacterial DNA using a 5ng/µl plant DNA solution (sample A1 in Table 1) as the solvent

Key Message

Bacterial contamination of *in vitro* plant cultures: confounding effects on somaclonal variation and detection of contamination in plant tissues

Plant Cell, Tissue and Organ Culture

Moreno-Vázquez Santiago [*]; Larrañaga Nerea; Uberhuaga Elizabeth C; Braga Eugenia Jacira Bolacel; Pérez-Ruíz César

* author for correspondence, Departamento de Biología Vegetal, E.T.S. Ingenieros Agrónomos, Universidad Politécnica de Madrid, 28040 Ciudad Universitaria, Madrid, Spain; e-mail santiago.moreno@upm.es

Key Message

Bacteria from the genus *Bacillus* are demonstrated to interfere with and confound somaclonal variation studies in plants. Genus-specific PCR markers for the main bacterial genera causing contamination in *in vitro* plant cultures (*Bacillus, Pseudomonas, Staphylococcus, Lactobacillus, Erwinia, Enterobacter* and *Xanthomonas*) are proposed and evaluated

Figure 1
Click here to download Figure: Fig1_Moreno-Vazquez_2014.doc

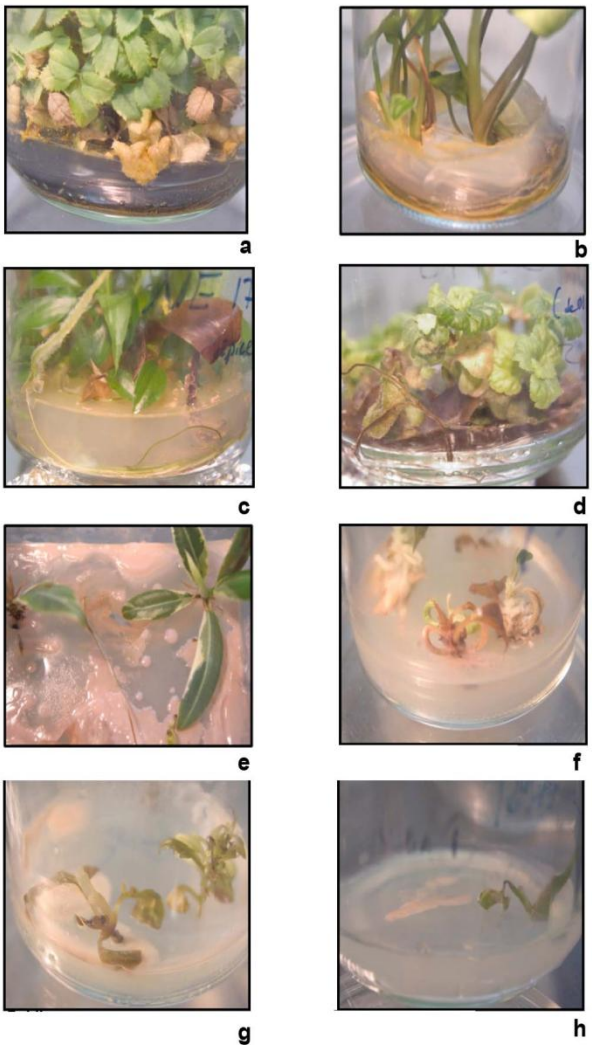


Figure 2

Click here to download Figure: Fig2_Moreno-Vazquez_2014.doc

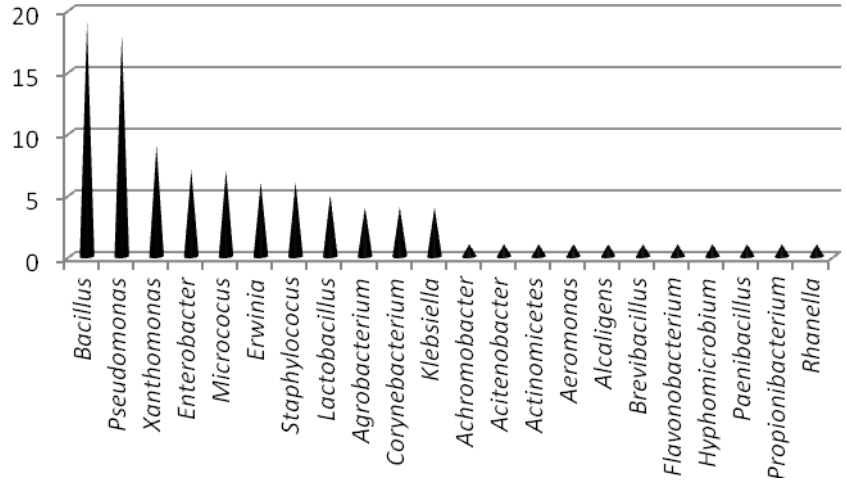


Figure 3

Click here to download Figure: Fig3_Moreno-Vazquez_2014.doc

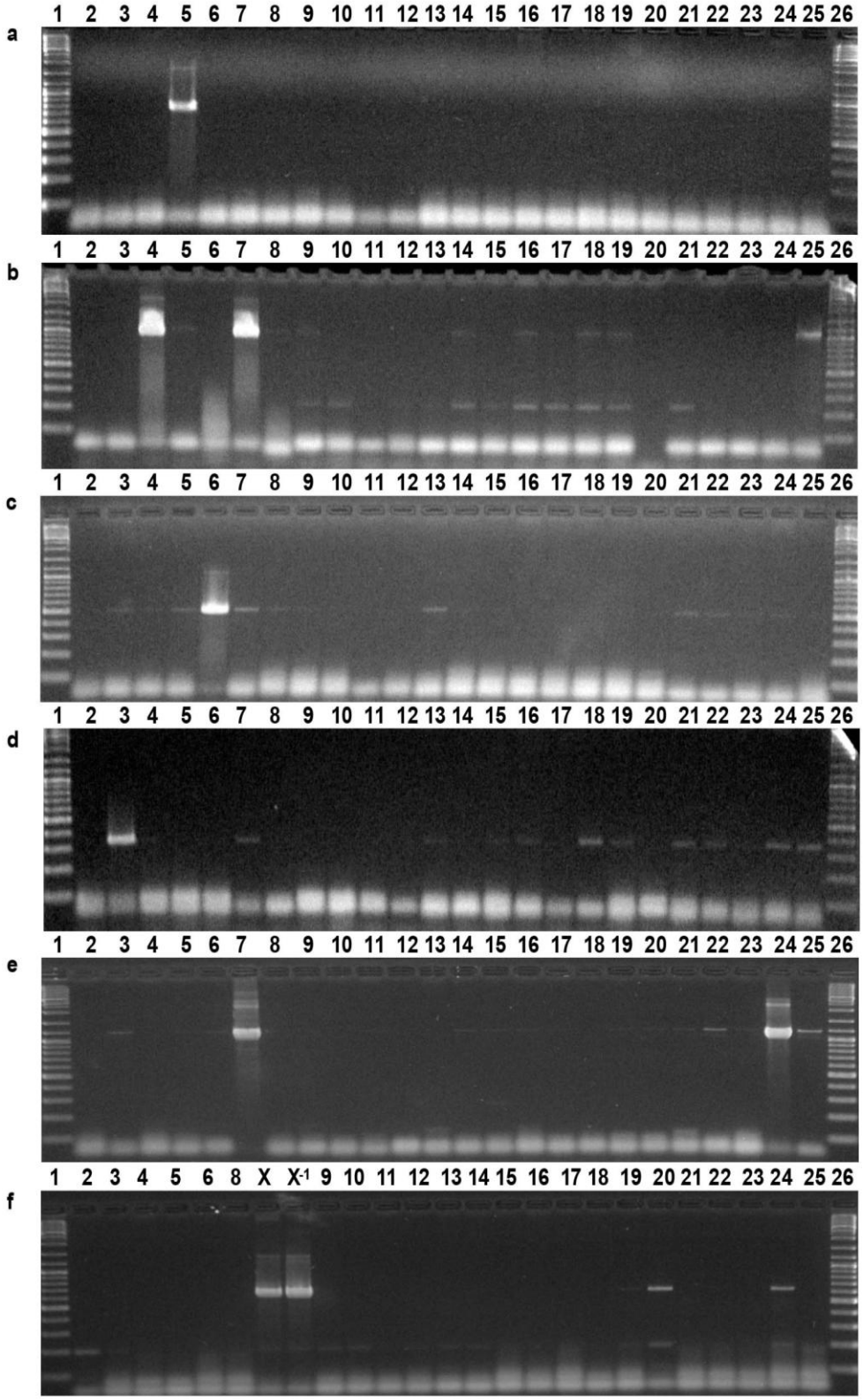
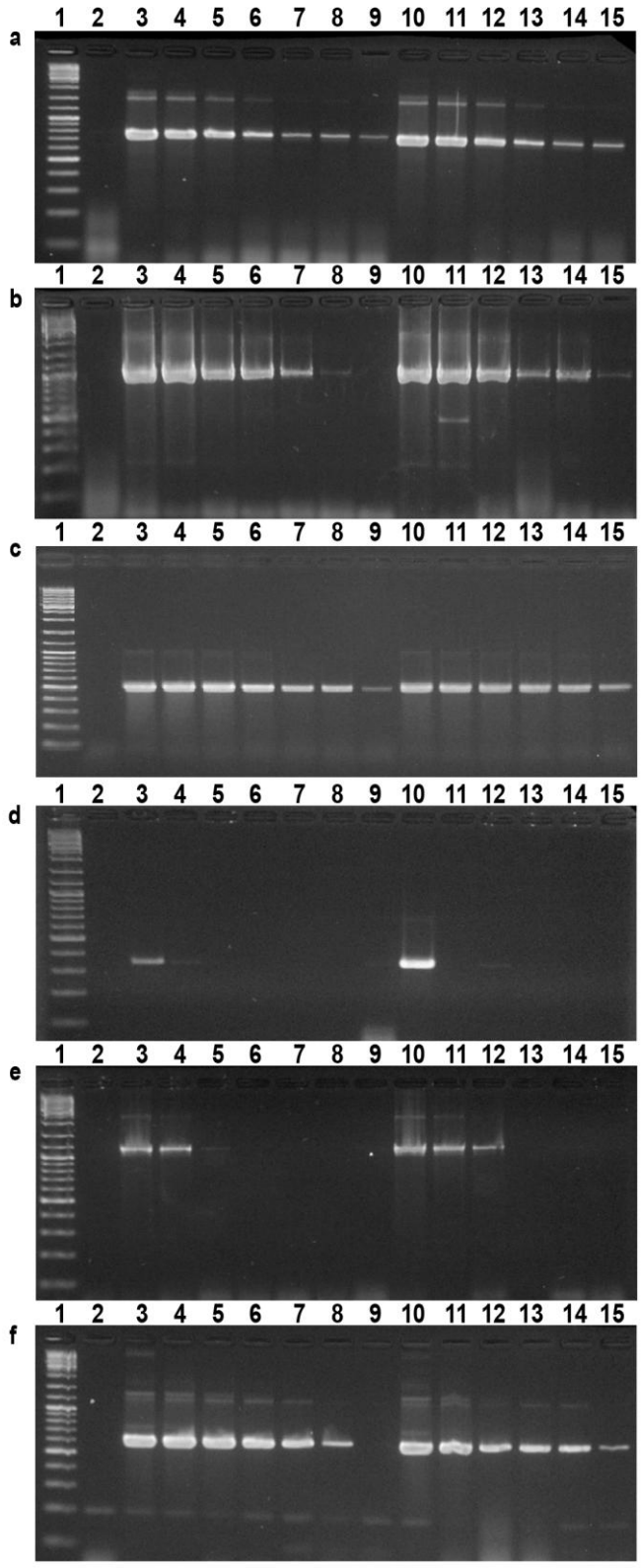


Figure 4

Click here to download Figure: Fig4_Moreno-Vazquez_2014c.doc



Bacterial contamination of *in vitro* plant cultures: confounding effects on somaclonal variation and detection of contamination in plant tissues

Plant Cell, Tissue and Organ Culture

Moreno-Vázquez Santiago [*]; Larrañaga Nerea; Uberhuaga Elizabeth C; Braga Eugenia Jacira Bolacel; Pérez-Ruíz César

* author for correspondence, Departamento de Biología Vegetal, E.T.S. Ingenieros Agrónomos, Universidad Politécnica de Madrid, 28040 Ciudad Universitaria, Madrid, Spain; e-mail santiago.moreno@upm.es

Table 1 Panel of *in vitro* plant cultures used for testing genus-specific PCR primers. Cultures A and B had no apparent symptoms of bacterial presence either on plant tissue or on gel and were considered control cultures. Cultures C to K showed apparent symptoms of bacteria only on gel. The bacterial species growing on growth medium were determined by direct PCR of colonies with universal primers FPL06 and RDR245 and subsequent sequencing as detailed in Material and Methods. For the identification of the bacterial species growing associated to plant tissues DNA extracts from two samples of plant tissue were analyzed: one sample close to the gel surface (subscript 1), one sample relatively far from the gel surface (subscript 2). Identification was carried out by PCR with bacterial genus-specific primers (see Table 2) and in some instances (Online Resource 3) confirmed by sequencing. MS, Murashige & Skoog; BAP, 6-benzylaminopurine; T, thiamine (hydrochloride); M, Myo-inositol; NAA, 1-naphtalene acetic acid; C, charcoal activated; TDZ, thidiazuron

***In vitro* cultures**			**Bacteria on gel**		**Bacteria associated to plant tissue**	
code	**plant species**	**media**	**color**	**species**	**DNA extract code**	**species**
A	*Dendranthema* sp.	MS 0.5 mg/l BAP 10 mg/l T 100 ml/l M 2 ml/l $AgNO_3$	---	---	A_1	undetected
B	*Pelargonium* sp.	MS 10 mg/l T 10 ml/l M 2 ml/l AgNO3	---	---	B_1	undetected
C	*Rosa* sp.	MS 0.2 mg/l BAP 0.25 NAA 1gr/l C	pale yellow	undetermined	C_1 C_2	undetected undetected
D	*Pelargonium* sp.	MS	pale yellow	undetermined	D_1 D_2	undetected undetected

E	*Spathiphyllum wallis*	MS 0.5 mg/l BAP	pale pink	*E. coli*	E_1 E_2	undetected undetected
F	*Dendranthema*	MS 0.1 mg/l BAP 10 mg/l T 100 ml/l M 2 ml/l AgNO3	pale pink	undetermined	F_1 F_2	undetected undetected
G	*Ficus elastica*	MS 10 mg/l T 100 ml/l M 2 ml/l AgNO3	white pink	*E. coli*	G_1 G_2	undetected *Xanthomonas*
H	*Ficus elastica*	MS 0.5mg/l BAP	white-pink	*E. coli*	H_1	undetected
I	*Dahlia* sp.	MS 0.5mg/l BAP 4 mg/l $AgNO_3$	white	*Bacillus* sp.	I_1 I_2	undetected undetected
J	*Spathiphyllum* sp.	MS 0.1 mg/l BAP 10 mg/l T 100 ml/l M 2 ml/l $AgNO_3$	white-pink	undetermined	J_1	*Enterobacter* sp. *Xanthomonas* sp.
K	*Ficus* sp.	MS 0.05 mg/l TDZ 10mg/l T 100 ml/l M	white	*Pseudomonas* sp.	K_1	*Pseudomonas* sp.

Table 2 Genus-specific PCR primer pairs for bacterial detection in plant *in vitro* cultures. Their specificity was determined analyzing the bacteria control panel described in Material and Methods. Primer sensitivity was determined under two conditions as described in Material and Methods: a) on serial dilutions of bacterial DNA using water as the solvent, b) on serial dilutions of bacterial DNA using plant DNA solutions as the solvent.

Name	Sense	Sequence 5´-3´	Annealing temp. (ºC)	Extension time	Expected size (bp)	Specificity	Sensitivity (ng/µl)	
							water	plant DNA solution
FB1	F	ACGATGCGTAGCCGAC	65	1m 15 s	787	*Bacillus*	5.10^{-5}	5.10^{-5}
RB1	R	CCATGCACCACCTGTCACTCT						
FP1	F	CATACGTCCTACGGGAGAAAGC	65	1 m 15 s	1105	*Pseudomonas*	5.10^{-5}	5.10^{-5}
RP2	R	CTCCACCTCGCGGCTTG						
FS2	F	CAACGCGAAGAACCTTACCAAATC	65	1 m 15 s	504	*Staphylococcus*	5.10^{-5}	5.10^{-5}
RS2	R	TCCCACCTTCGACGGCTAG						
FL1	F	GGCAGCAGTAGGGAATCTTCCA	68	1 m	349	*Lactobacillus*	5	5
RL1	R	GCATTTCACCGCTACACATGGAG						
FE1	F	GCGGACGGGTGAGTAATGTC	70	1 m 15 s	1190	*Erwinia, Enterobacter*	5.10^{-1}	5.10^{-2}
RE1	R	GCACTTTATGAGGTCCGCTTGCT						
FX1	F	CTCTTTCGTGGGGGATAACGTAG	65	1 m 15 s	630	*Xanthomonas*	5.10^{-5}	5.10^{-5}
RX1	R	CGTGCCTCAGTGTCAGTGTTG						

Bacterial contamination of *in vitro* plant cultures: confounding effects on somaclonal variation and detection of contamination in plant tissues

Plant Cell, Tissue and Organ Culture

Moreno-Vázquez Santiago [*]; Larrañaga Nerea; Uberhuaga Elizabeth C; Braga Eugenia Jacira Bolacel; Pérez-Ruíz César

* author for correspondence, Departamento de Biología Vegetal, E.T.S. Ingenieros Agrónomos, Universidad Politécnica de Madrid, 28040 Ciudad Universitaria, Madrid, Spain; e-mail santiago.moreno@upm.es

Online Resource 1 PCR primers for somaclonal studies on chrysanthemum

Name	Sequence 5´-3´	Target	
		Type of sequence	Domain
N-PDT1	GCAAGTGTCGTCTTCC	TIR-NBS-LRR	NB P-loop
N-PDT2	GCAAGTGTCGTCTTCC	TIR-NBS-LRR	NB P-loop
N-PDT3	GCAAGTGTCGTTTTCC	TIR-NBS-LRR	NB P-loop
N-PDT4	GCAAGTGTCGTTTTCC	TIR-NBS-LRR	NB P-loop
N-PDT5	GCAAGTGTCGTCTTICCCA	TIR-NBS-LRR	NB P-loop
N-PDT6	GCAAGTGTTGTCTTICCCA	TIR-NBS-LRR	NB P-loop
N-PHNT1	AGAGTAGTCTTACCACCA	CC-NBS-LRR	NB P-loop
N-PHNT2	AGAGTGGTTTTTCCATC	CC-NBS-LRR	NB P-loop
N-PLNT1	AGTCTTCCCCACTCCA	CC-NBS-LRR	NB P-loop
N-HDT1	GAGGGCGAGGGGGA	TIR-NBS-LRR	NB Hydrofobic
N-HDT2	GAGGGCGAGGGGAA	TIR-NBS-LRR	NB Hydrofobic
N-HDT3	GAGGGCGAAGGGGA	TIR-NBS-LRR	NB Hydrofobic
N-HDT4	GAGGGCTAAGGGAAG	TIR-NBS-LRR	NB Hydrofobic
N-HDT5	GAGTGCTAATGGAAGACC	TIR-NBS-LRR	NB Hydrofobic
N-HDT6	GAGGGCGAGGGGAA	TIR-NBS-LRR	NB Hydrofobic
N-HDT7	CAGAGCTAGGGGGAGT	TIR-NBS-LRR	NB Hydrofobic
N-HDT8	CAAAGTCAATGGAAGTCC	TIR-NBS-LRR	NB Hydrofobic
N-HHT9	AAGTGCTAATGGGAAC	TIR-NBS-LRR	NB Hydrofobic
N-HDNT1	AGAGCTAAGGGCAGTC	CC-NBS-LRR	NB Hydrofobic
N-HDNT2	AAAGCTAAGGGCAGTC	CC-NBS-LRR	NB Hydrofobic
N-HDNT3	ATAGCTAACGGCAGG	CC-NBS-LRR	NB Hydrofobic
N-HDNT4	AGAGCTAAGGGCAAC	CC-NBS-LRR	NB Hydrofobic
N-HDNT5	AAAGCTAAGGGCAATCC	CC-NBS-LRR	NB Hydrofobic
N-HDNT6	ATAGCTAAGGGCAATCC	CC-NBS-LRR	NB Hydrofobic
N-HDNT7	TATTGCAAGAGGAACTCC	CC-NBS-LRR	NB Hydrofobic
N-HDNT8	GGCTGCTAGAGGCACA	CC-NBS-LRR	NB Hydrofobic
N-HHNT9	CAAAGCCAAAGGCAAA	CC-NBS-LRR	NB Hydrofobic
N-HLNT10	TATGGCAATAGGTAGACC	CC-NBS-LRR	NB Hydrofobic
M-ALI-CAP1	CTWCCAAACGACTC	class 2 mobile element	
M-ALI-CAP2	TWCCAAACGAGTC	class 2 mobile element	
M-ALI-MA	CTWCCAAACGACC	class 2 mobile element	
M-hATpin1	TCCACCCCTA	class 2 mobile element	
M-hATpin2	TTTTACACCCCTA	class 2 mobile element	
M-hATpin3	TTGAACACCCCTA	class 2 mobile element	
M-LTR-P2	TGTATTAGAATATCA	class 1 mobile element	
M-LTR-P1	GTTCCAGTCAAAGGCAAGTC	class 1 mobile element	
M-LTR-P1ci	GACTTGCCTTTGACTGGAAC	class 1 mobile element	
M-LTR-P2ci	TGATATTCTAATACA	class 1 mobile element	
M-LTR-TA1	TGTTGGAGTTATGAT	class 1 mobile element	
M-LTR-TA1ci	ATCATAACTCCAACA	class 1 mobile element	
M-LTR-V	TGTTAGCTGTATATA	class 1 mobile element	
M-LTR-Vci	TATATACAGCTAACA	class 1 mobile element	
M-SINE1	GTTCGAGCTGTG	class 1 mobile element	
M-SINE2	GTTCGACTCGCG	class 1 mobile element	
M-TRIM1	CCTAACTCACA	class 1 mobile element	
M-TRIM1ci	TGTGAGTTAGG	class 1 mobile element	
M-TRIM2	ATCTCATTAATCACC	class 1 mobile element	
M-TRIM2ci	GGTGATTAATGAGAT	class 1 mobile element	
M-TRIM3	TTTGGGGTTGRGTTAGGCC	class 1 mobile element	
M-TRIM3ci	GGCCTAACYCAACCCCAAA	class 1 mobile element	
M-TRIM4	TTTGGGGTGARTTAGGCC	class 1 mobile element	
M-TRIM4ci	GGCCTAAYTCACCCCAAA	class 1 mobile element	
M-TRIM5	GTGGRACTTTTGTCATTCTTT	class 1 mobile element	
M-TRIM5ci	AAAGAATGACAAAAGTYCCAC	class 1 mobile element	
M-STOW1	TTGGAACAGAGGGAG	class 2 mobile element	
M-STOW2	TTTTCTTAACTCCGT	class 2 mobile element	

Online Resource 2 List of references reviewed on the use of unspecific PCR for the detection of somaclonal variants in plant *in vitro* cultures

Afrasiab H, Iqbal J (2012) Genetic analysis of somaclonal variants and induced mutants of potato (*Solanum tuberosum* L.) cv. Diamant using RAPD markers. Pak J Bot 44:215-220

Afza R, Xie J, Shen M, Zapata-Arias F, Fundi H, Lee K, Bobadilla-Mucino E, Kodym A (2001) Detection of androclonal variation in anther-cultured rice lines using RAPDs. In Vitro Cell Dev Biol -Plant 37:644-647

Albani M, Wilkinson M (1998) Inter simple sequence repeat polymerase chain reaction for the detection of somaclonal variation. Plant Breed 117:573-575

Bairu M, Fennell C, van Staden J (2006) The effect of plant growth regulators on somaclonal variation in Cavendish banana (*Musa* AAA cv. 'Zelig'). Sci Hortic 108:347-351

Brown PTH, Lange FD, Kranz E, Lörz H (1993) Analysis of single protoplast and regenerated plants by PCR and RAPD technology. Mol Gen Genet 237:311-317

Chandrika M, Rai VR, Thoyajaksha (2010) ISSR marker based analysis of micropropagated plantlets of *Nothapodytes foetida*. Biol Plant 54:561-565

Chandrika M, Thoyajaksha, Rai VR, Kini KR (2008) Assessment of genetic stability of *in vitro* grown *Dictyospermum ovalifolium*. Biol Plant 52:735-739

Devarumath R, Nandy S, Rani V, Marimuthu S, Muraleedharan N, Raina S (2002) RAPD, ISSR and RFLP fingerprints as useful markers to evaluate genetic integrity of micropropagated plants of three diploid and triploid elite tea clones representing Camellia sinensis (China type) and C-assamica ssp assamica (Assam-India type). Plant Cell Rep 21:166-173

Gostimsky S, Kokaeva Z, Konovalov F (2005) Studying plant genome variation using molecular markers. Russ J Genet 41:378-388

Hsu T, Tsai W, Wang D, Lin S, Hsiao Y, Chen W, Chen H (2008) Differential gene expression analysis by cDNA-AFLP between flower buds of *Phalaenopsis* Hsiang Fei cv. H. F. and its somaclonal variant. Plant Science 175:415-422

Kaity A, Ashmore SE, Drew RA, Dulloo ME (2008) Assessment of genetic and epigenetic changes following cryopreservation in papaya. Plant Cell Rep 27:1529-1539

Leroy X, Leon K, Hily J, Chaumeil P, Branchard M (2001) Detection of *in vitro* culture-induced instability through inter-simple sequence repeat analysis. Theor Appl Genet 102:885-891

Li X, Yu X, Wang N, Feng Q, Dong Z, Liu L, Shen J, Liu B (2007) Genetic and epigenetic instabilities induced by tissue culture in wild barley (*Hordeum brevisubulatum* (Trin.) Link). Plant Cell Tissue Organ Cult 90:153-168

Mandolino G, DeMarco S, Faeti V, Bagatta M, Carboni A, Ranalli P (1996) Stability of fingerprints of *Solanum tuberosum* plants derived from conventional tubers and vitrotubers. Plant Breeding 115:439-444

Ngezahayo F, Dong Y, Liu B (2007) Somaclonal variation at the nucleotide sequence level in rice (*Oryza sativa* L.) as revealed by RAPD and ISSR markers, and by pairwise sequence analysis. J Appl Genet 48:329-336

Palombi MA, Lombardo B, Caboni E (2007) In vitro regeneration of wild pear (*Pyrus pyraster* Burgsd) clones tolerant to Fe-chlorosis and somaclonal variation analysis by RAPD markers. Plant Cell Rep 26:489-496

Phong D, Muoi L, Binh L (2001) RAPD variability in rice (*Oryza sativa* L.) plants derived from desiccation-tolerant calli. Euphytica 121:297-303

Rasheed S, Fatima T, Husnain T, Bashir K, Riazuddin S (2005) RAPD characterization of somaclonal variation in indica basmati rice. Pak J Bot 37:249-262

Rzepka-Plevnes D, Kulpa D, Smolik M, Glowka M (2007) Somaclonal variation in tomato *L. pennelli* and *L. peruvianum* f. *glandulosum* characterized in respect to salt tolerance. J Food Agric Environ 5:194-201

Saker M, Adawy S, Mohamed A, El-Itriby H (2006) Monitoring of cultivar identity in tissue culture-derived date palms using RAPD and AFLP analysis. Biol Plant 50:198-204

Salvi N, George L, Eapen S (2001) Plant regeneration from leaf base callus of turmeric and random amplified polymorphic DNA analysis of regenerated plants. Plant Cell Tissue Organ Cult 66:113-119

Thomas J, Vijayan D, Joshi S, Lopez S, Kumar R (2006) Genetic integrity of somaclonal variants in tea (*Camellia sinensis* (L.) O Kuntze) as revealed by inter simple sequence repeats. J Biotechnol 123:149-154

Villordon A, Labonte D (1995) Variation in randomly amplified DNA markers and storage root yield in Jewel sweet-potato clones. J Am Soc Hort Sci 120:734-740

Vroh-Bi I, Anagbogu C, Nnadi S, Tenkouano A (2011) Genomic characterization of natural and somaclonal variations in bananas (*Musa* spp.). Plant Mol Biol Rep 29:440-448

Online Resource 3 Nucleotide sequences of 16S rRNA fragments from contaminant bacteria in plant *in vitro* cultures. Both bacteria growing on the gel surface and bacterial growing in plant tissue were considered

Culture code (type of sample)	Nucleotide sequence (5´- 3´)	Homology (BLASTN)
J1 (plant tissue)	CTTCGGGCCTCGCGCGGTTGGATGGACCGATGTTCGATTAGCTAGTTG GTAGGGTAATGGCCTACCAAGGCGACGATCGATAGCTGGTCTGAGAG GATGATCAGC	*Rhodanobacter (Xanthomonaceae)*
J1 (plant tissue)	AGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGG GCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGG GTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGA CCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA GCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGC CGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGG AGGAAGGCGATAAGGTTAATAACCTTGTCGATTGACGTTACCCGCAG AAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCG GTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGC ATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCA GGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG AAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTG GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT GTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACG CGTTAAGTCGACCG	*Enterobacter*
K1 (plant tissue)	GCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCG ACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGA GACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA CAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGT CTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAA:GGGTACTTACCTA ATACGTGAGTATTTTGACGTTACCGACAGAATAAGCACCGGCTAACT CTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGA ATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTG AAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTA GAGTATGGTAGAGGGTGGTGGAATTT	*Pseudomonas*
E, G, H, U, V, Y (colonies on gel)	AGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAA GTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCG CATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAG TGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCG GAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCC	*Escherichia coli*
I (colony on gel)	CTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGT CAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATG GATGGTACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCATA AAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAG CCGGAATCGCTAGTAATCGCGGATCAG:CATGCCGCGGTGAATACGTT	*Bacillus*
K, L (colonies on gel)	CTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGT CAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATG GTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAA AACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGT CGGAATCGCTAGTAATCGCGAATCAG:AATGTCGCGGTGAATACGTT	*Pseudomonas*
M (colony on gel)	CTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGT CAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTACTACAATG GTCGGTACAGAGGGTTGCGATACCGCGAGGTGGAGCCAATCCCAGA AAGCCGATCCCAGTCCGGATCGAAGTCTGCAACTCGACTTCGTGAAG TCGGAATCGCTAGTAATCGCGGATCAGCTATGCCGCGGTGAATACGT T	*Rhodanobacter*
N (colony on gel)	CNTGGGATACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGNCNT CAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGG CCGGTACAAAGGGCTGCAATACCGCGAGGTGGAGCGAATCCCAAAA AGCCGGTCCCAGTTCGGATTGAGGTCTGCAACTCGACCTCATGAAGT CGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTT	*Microbacterium oxydans*

O, P, Q (colonies on gel)	CATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGNNNNNNN NNAATCATCATGCCCCTTATGTCTTGGGCTTCACGNATGCTNCAATGG CCGGTACAAAGGGCTGCGATACCGTAAGGTGGAGCGAATCCCAAAA AGCCGGTCTCAGTTCGGATTGAGGTCTGCAACTCGACCTCATGAAGT CGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTT	*Curtobacterium/Bacillus*
S, T (colonies on gel)	CATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGT CAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGG CCGGTACAAAGGGCTGCGATACCGTAAGGTGGAGCGAATCCCAAAA AGCCGGTCTCAGTTCGGATTGAGGTCTGCAACTCGACCTCATGAAGT CGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTT	*Curtobacterium/Bacillus*
R, W (colonies on gel)	CTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGT CAAATCATCATGCCCCTTATGACCTGGGCTACACACGTACTACAATG GCCGGTACAACGGGCAGTGAAACCGCGAGGTGGAACGAATCCTAAA AAGCCGGTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAG TCGGAATTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTT	*Paenibacillus*
X (colony on gel)	CTAAGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGT CAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGG ACAATACAAAGGGCAGCGAAACCGCGAGGTCAAGCAAATCCCATAA AGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGACTATATGAAGCT GGAATCGCTAGTAATCGTAGATCAG:CATGCTACGGTGAATACGTT	*Staphylococcus*

Bacterial contamination of *in vitro* plant cultures: confounding effects on somaclonal variation and detection of contamination in plant tissues

Plant Cell, Tissue and Organ Culture

Moreno-Vázquez Santiago [*]; Larrañaga Nerea; Uberhuaga Elizabeth C; Braga Eugenia Jacira Bolacel; Pérez-Ruíz César

* author for correspondence, Departamento de Biología Vegetal, E.T.S. Ingenieros Agrónomos, Universidad Politécnica de Madrid, 28040 Ciudad Universitaria, Madrid, Spain; e-mail santiago.moreno@upm.es

M-V. S. conceived, designed and supervised the experiments and wrote the paper; L. N. performed experiments for bacterial detection and identification and wrote the paper; U. E. C. obtained most *in vitro* plant cultures and edited the paper; B. E. J. B., performed experiments for somaclonal variation detection and edited the paper; P-R. C. gave technical support and conceptual advice and wrote the paper.

Dr. Sergio J Ochatt
Editor-in-Chief
Plant Cell, Tissue and Organ Culture (PCTOC): Journal of Plant Biotechnology

Dear Dr. Ochatt:

We really appreciate your comments as well as those from the associate editor and the two reviewers regarding the manuscript submitted to *PCTOC* entitled "Bacterial contamination of *in vitro* plant cultures: confounding effects on somaclonal variation and detection of contamination in plant tissues". Your suggestions have been very helpful for increasing the clarity and readability of our manuscript.

We have tried to address all the issues raised, starting with the language usage. To improve the English, AJE (http://www.aje.com/) provide a professional edit. Please, find enclosed an AJE certificate.

In the next few pages, we try to carefully address all queries and comments by the associate editor and reviewers. After each comment by the reviewers (boldface), we wrote our response (italics).

Thank you again for your time and for considering our manuscript.

Sincerely,

Santiago Moreno-Vázquez

Associate Editor.

Line 51: do not produce obvious symptoms and are propagated

This has been modified in the text.

Line 82: recipients? do you mean explants and culture vessels?

This has been modified in the text ('culture vessels' instead of 'recipients').

In the methods section please identify each item that is abbreviated the first time it is used. Identify MS and cite the article, NAA, BA, etc. all need to be spelled out the first time they are used.

This has been modified in the text.

Line 92. MS Medium - is the only one used, media is plural.

Solid MS plant growth media with species-dependent additives was used (Table 1)

Solid MS-based plant growth media with species-dependent additives was used (Table 1)

This has been modified in the text. This was a common mistake in the manuscript, and we corrected it in all cases.

Line 137. growth medium not growing medium.

This has been modified in the text.

Line 142-3. These are cultivars (cultivated varieties), not varieties (a specific botanical term).

This has been modified in text.

Lines 230-234: There are several papers concerning methods to detect bacteria and fungi in tissue cultured plants. See articles by M. Kane, P. Buckley, P. Tanprasert and B. Reed since 1990.

The associate editor is right. We modified lines 230-234 as follows:

'(. . .). However, beyond purification using standard bacteriological methods, culture indexing systems and morphological and biochemical tests, there are no reports in the literature on molecular methods to specifically detect bacteria contaminants in in vitro plant cultures. (. . .)'

Pseudomonas is commonly found on plant surfaces of certain genera, so it is also likely to be a contaminant in cultures (see Tanprasert and Reed 1997).

This has been modified in the text.

Reviewer #1

My only question is whether the somaclonal variation in chyranthemeum that was observed in the 7 and 9 subculture also had a phenological manifestation?

Do you mean phenotypical instead of phenological manifestation? We did not notice any difference between 7 and 9 subcultures and other subcultures of RR 11.

I have a problem with the use of the word "recipient" in the text. I guess in means "Culture vessel"?

This has been modified in the text.

Reviewer #2

The authors should also mention that the bacterial identification could assist in developing methods for decontamination.

Excellent point! We have modified the manuscript to include the following sentence:

"The identification of the bacteria in in vitro plant cultures has an additional advantage: when using antibiotics for decontamination, the selection of the most efficient compound can be

refined using this information. Choosing the appropriate antibiotic will improve decontamination and reduce the secondary effects of their use on plants."

1. Lines 205-208: "the tissue sample from the eighth generation used for DNA extraction did not appear to contain bacteria but the tissue sample from the eighth generation used to produce the ninth generation did very likely contain bacteria."

Another explanation could be that the 8th generation became contaminated during the process of producing the ninth generation. Bacterial contaminants can generally be detected during the initial culture, it is hard to imagine that there was no contamination from 1st to 8th generations, but contamination suddenly occurred in the 9th generation.

Apparently, the first generation exhibiting Bacillus contamination was the 7th generation. However, the second generation with Bacillus contamination was not the 8th generation, but the 9th generation. We do not believe contamination suddenly occurred in the 9th generation. Instead, we hypothesized that the 9th generation inherited contamination from the 8th generation, which in his turn inherited contamination from the 7th generation. This hypothesis would be possible only if we assume that the tissue sample from the 8th generation was only locally contaminated; that is, the part used for DNA extraction was free of bacteria, but the part used to produce the 9th generation was contaminated with bacteria.

2. Lines 212-215: "We can conclude that in somaclonal variation studies performed with non-specific (RAPDs, ISSR, AFLP) or semi-specific PCR, it would be always advisable to determine the sequence of any candidate polymorphic DNA to discard interferences from contaminant bacteria.".

Somaclonal variants are generally identified based on morphological changes when regenerated plantlets are grown in greenhouses, or occasionally in culture vessels. Samples taken from those plants are then used for molecular marker analysis. Because plants are grown ex vitro, samples may harbor bacteria and other microorganism. Molecular marker analysis often includes control plant materials to identify false bands. The authors' statement per se may be correct. In reality, however, it is hardly applicable.

The chrysanthemum plant material we analyzed for somaclonal variation was never grown ex vitro except for the mother plant. We never noticed any visual differences among them. Despite this, we decided to look for molecular differences in areas of the genome that have been described as being prone to changes under stress: NB-LRR resistance genes regions and transposable element rich regions.

When using non-specific or semi-specific molecular markers (RAPDs, AFLP, etc.), even using control plants (mother plants should be considered control plants), the nature (true somaclonal variation or bacterial contamination) of the novel bands will be uncertain unless they are sequenced.

3. Lines 240-242: "This might be due to the amplification of contaminating bacterial 16S rDNA fragments in the PCR reagents (Grahn et al. 2003; Chang et al. 2011)."

Lines 250-251: "The reason why Pseudomonas is also frequently found as a contaminant in in vitro plant cultures is unknown."

Any possibility of cultural environment being contaminated?

The polymerase supplier (Biotools) confirmed that the polymerase we have been using (ref. 10.042) may contain contaminant E. coli DNA. This is probably the simplest explanation for the results using universal primers for bacteria. It is unlikely a massive bacterial contamination from the lab environment (mainly laminar flow hood) because no one in this lab is manipulating E. coli or any other bacteria, and as a general rule, contaminated culture vessels are immediately separated, sterilized and discarded.

1. Abstract should specify materials and methods used in this study, key results, and their implications.

This has been modified in the text.

2. Lines 77-92: Individual plant materials should be mentioned in this section.

This has been modified in the text.

3. Lines 191-193: It will be helpful if a gel electrophoresis profile is presented to show the three bands.

We decided not to show these bands because we think they are not necessary for understanding the results we are presenting. Including these bands will require adding three extra images (one per band), which will enlarge the manuscript.

4. Figure 4. Lines 2 and 9 in the gel electrophoresis profile were control water. It appeared that line 9 has a band in panel a and c. Please explain.

First of all, it is important to note a mistake in the caption of figure 4. Lane 9 is not actually control water but control plant DNA (sample A1 in Table 1).

Initially, we thought that the bands mentioned by the reviewer are a consequence of using a DNA polymerase contaminated with E. coli. The polymerase supplier (Biotools) confirmed that the polymerase we have been using (ref. 10.042) might contain contaminant E. coli DNA. However, we believe that contaminant E. coli is not likely the reason for those bands because neither the primer pair specific for Bacillus (Fig. 4a) nor the pair for Staphylococcus (Fig. 4c) would amplify template DNA from E. coli. The primer design ensures too many mismatches when aligning them with E. coli 16S rDNA sequences.

After the reviewer comments, we repeated PCRs corresponding to figures 4a and 4c. The pictures taken after agarose electrophoresis did not change things too much except for figure 4c, where control water did not produce any amplification at all. We prepared a new figure 4c including the picture with the new results. Still an important question needs to be addressed: Why specific primers for Bacillus and Staphylococcus were able to amplified control plant DNA, but no amplification was obtained with specific primers for other bacteria? Assuming that neither cpDNA nor mitochondrial DNA extracted from plant sample A1 would produce

amplifications with any of these primers, the easiest answer to this question is to accept that A1 contained a very small amount of an asymptomatic bacteria closer to Bacillus and Staphylococcus than to any of the other bacteria considered in this study.

We have modified the manuscript to include the following sentence:

'(. . .). However, when using control plant DNA (A1 in Table 1, lane 9 in Figure 4) specific primers for Bacillus and Staphylococcus produced bands of the expected size but very faint compared to bands obtained using serial dilutions as templates. We assume that the primer design ensures that neither cpDNA or mitochondrial DNA extracted from plant sample A1, nor E. coli DNA introduced with the DNA polymerase would serve as templates to produce amplifications with any of the bacterial primers. Then, the easiest explanation for these bands could be that they were caused by a very small amount of an asymptomatic bacteria (closer to Bacillus and Staphylococcus than to any of the other bacteria considered in Table 2) present in the plant sample A1. (. . .)'

AMERICAN JOURNAL EXPERTS

EDITORIAL CERTIFICATE

This document certifies that the manuscript listed below was edited for proper English language, grammar, punctuation, spelling, and overall style by one or more of the highly qualified native English speaking editors at American Journal Experts.

Manuscript title:

Bacterial contamination in plant in vitro cultures: confounding effects on somaclonal variation and detection in plant tissues

Authors:

Moreno-Vázquez Santiago, Larrañaga Nerea, Uberhuaga Elizabeth C, Braga Eugenia Jacira Bolacel, Pérez-Ruíz C

Date Issued:

June 5, 2014

Certificate Verification Key:

F1A9-6AA0-B8F9-3AF3-64DE

This certificate may be verified at www.aje.com/certificate. This document certifies that the manuscript listed above was edited for proper English language, grammar, punctuation, spelling, and overall style by one or more of the highly qualified native English speaking editors at American Journal Experts. Neither the research content nor the authors' intentions were altered in any way during the editing process. Documents receiving this certification should be English-ready for publication; however, the author has the ability to accept or reject our suggestions and changes. To verify the final AJE edited version, please visit our verification page. If you have any questions or concerns about this edited document, please contact American Journal Experts at support@aje.com.