Development of PCR-based molecular marker for screening of disease-suppressive composts against Fusarium wilt of tomato (*Solanum lycopersicum* L.)

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Abstract

The present study was carried out to develop a PCR-based molecular marker suitable for screening of disease-suppressive composts against Fusarium wilt of tomato. An effective uncultured bacterial community was screened from our previous study on investigation of microbial communities in composts for their potential for biocontrol of Fusarium wilt. Based on available sequence information (Accession no. HQ388491) of selective community, PCR-based molecular markers were designed and tested for their specificity in different compost sample. To confirm specificity of designed marker, real-time reverse transcription-PCR (qRT-PCR) analysis was performed. Selective marker efficacy was further tested for different set of composts and results were cross-verified by conducting bioassay of same composts against Fusarium wilt in tomato crop. Results showed that out of two designed set of primers (i.e., PAC1F/PAC1R and PAC4F/PAC4R), primer set PAC4F/PAC4R resulted in successful amplification of 199 bp in highly disease-suppressive compost (i.e., CPP); however, no/below detection level amplification was observed in non-suppressive compost (JC). qRT-PCR analysis confirmed the specificity of selective marker by representing single peak in melting curve. A clear difference was observed in relative population of selective community in different set of composts. It was observed maximum in the most effective compost, i.e., CPP followed by other disease-suppressive composts. Cross-examination of results with bioassay confirmed that composts with presence of selective bacterial community having no/very less disease incidence of Fusarium. It is clearly evident from the study that such kind of molecular markers can be developed and used in future research focusing on compost-based disease suppression.

Keywords Compost · Disease suppression · PCR · Molecular marker · qRT-PCR · Fusarium wilt

Abbreviations

PCR Polymerase chain reaction
qRT-PCR Real-time reverse transcription-PCR

Introduction

Every year, plant diseases cause significant economic crop loss all over the world (Koenning and Wrather 2010; Chakraborty and Newton 2011; Siebold and Tiedemann 2013; Chellemi et al. 2016). Among all plant diseases, *Fusarium* spp. is the most common species comprising pathogens, parasites and saprophytes, affecting all vegetative and reproductive parts of plants (Jennings et al. 2004; Klein et al. 2011; Khastini et al. 2012; Akhter et al. 2015; Fu et al. 2017). Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops worldwide that could be used in many different ways like fresh as well as processed (Radzevičius et al. 2009; Glogovac et al. 2010; Gupta et al. 2017). *Fusarium oxysporum* f. sp. *lycopersici* is one of the most devastating pathogens of tomato (Balaž et al. 2009; Boukerma et al. 2017), causing great losses in productivity and fruit quality, especially on sensitive varieties under

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favorable weather conditions and once contaminated, soil remains contaminated indefinitely (Agrios 2005).

The most significant control for *Fusarium* wilt of tomato was use of methyl bromide, but due to environmental and human health concern, application of methyl bromide in fruits and vegetable crops has been banned by Montreal protocol (Minuto et al. 2006). Therefore, compost was considered as a potential alternative of methyl bromide to suppress disease, production of fruit and vegetable crops (Salman et al. 2017). The composts from various sources are well known for their role in suppression of plant diseases and are commonly used as a natural biological control since long time (Grebus et al. 1994; Hoitink et al. 1997; Vestberg et al. 2009; Hadar 2011; Shivlata and Satyanarayana 2017). The disease suppression of compost is considered as a result of two components: one when compost acts as a source for various biochemical compounds such as hormones, antibiotics and other secreted chemicals of microorganism, and secondly the beneficial microbes of compost (Özer and Köycü 2006; Fuchs 2010; Mehta et al. 2016; Scotti et al. 2016). However, all the composts are not equally effective towards disease suppression since they carry a huge difference in biochemical and microbial activities (Mupambwa et al. 2016; Tossavainen et al. 2017). It is known that intense microbial activity is observed in decomposition of most biodegradable materials during composting process (Weltzien 1991; Adani et al. 1997) and the majority of characteristics of compost are due to the presence of microbial activity. Judging the disease suppression capability of compost based on presence of these biochemical compounds is a costly affair, as it involves highly sophisticated instruments like HPLC, GC–MS, etc. (Matsunaga et al. 2013; Fels et al. 2014). Microbe secretes majority of these useful biomolecules during the composting, so if we can detect presence of the beneficial microbes in the compost, we can correlate it to disease suppression.

Due to the lack of well-defined characteristics of compost and the non-availability of methods to assess it, there is an immediate need of development of quick detection tools to check the disease-suppressing capacity of compost. There are many studies where the researchers have explored the microbial diversity of different compost and soil by using molecular tools (Nannipieri et al. 2003; Mehta et al. 2014; Pudasaini et al. 2017). Differential gradient gel electrophoresis (DGGE) is one of the most commonly used techniques in diversity studies (Yu et al. 2015; Mehta et al. 2016). Previous studies have implied that molecular biology approaches could be effectively used for improved understanding of disease suppression by compost (Blaya et al. 2016; Ros et al. 2017). In previous studies, our laboratory has identified few types of compost that suppressed the occurrence of *Fusarium* wilt in tomato. Through DGGE profiling of compost, specific bacterial community was observed in highly disease-suppressive

compost (Mehta et al. 2016). Based on available information, this study was carried out to develop closely linked PCR-based molecular markers that can be used to detect specific microbial community in composts. Results of this study will be useful for marker-assisted study for disease suppressiveness property of different sets of compost.

Materials and methods

Compost collection and bioassay

In present study, seven composts [viz. Vermi Nagla (VN); Vermi Haldachaur (VH); Vermi Haldachaur Mature (VHM); BN: Bamboo NADEP (BN); Raw NADEP (RN); Compost with *Trichoderma* (CT) and Vermi Khanna (VK)] produced aerobically and collected from nearby areas of Pantnagar, Uttarakhand, (India) and screened for their disease suppressiveness property against *Fusarium* wilt (*Fusarium oxysporum* f. sp. *lycopersici*) of tomato. Similar methodology (Mehta et al. 2016) was used to conduct bioassay of selected composts. The composts were mixed at the rate of 20% (w/w) with autoclaved sand and filling 150 g of this potting mix in small pots. Inoculum of pathogen was produced in liquid potato dextrose medium and to infest the plants 12 ml of 5-day-old cultures (10^7 propagules per ml) was used. Equal number of control pots with same quantity of inoculum were used and experiment was replicated three times under controlled conditions in glass house. Supplementary light was provided by cool white lamps, $400 \mu\text{E m}^{-2} \text{s}^{-1}$, 400–700 nm, with a 16/8 h day/night cycle at 25/19 °C and 50% relative humidity.

DNA extraction from composts

DNA from compost was extracted using a HiPurA™ Soil DNA Kit (HiMedia, India) according to the manufacturer's instructions. Briefly, the cell lysis was performed by soil lysis solution (SL) in bead beating tubes and centrifuged at $13,000\times g$ for 1 min. Supernatant was collected and followed by addition of inhibitor removal solution (IRIS) and binding solution (SB) and centrifugation steps. Lysate was loaded into spin column included in the kit and centrifuged. Washing of DNA was performed with the washing solution (WSP) and finally DNA was eluted by adding 100 μl of elution buffer (ET) included in the kit. The extracted DNA was then stored at -20°C for further analysis. DNA concentration was estimated by measuring the absorbance at 260 nm/280 nm.

Development of specific primer

Nucleotide sequence of 16S rDNA gene from the disease suppressive compost in previous study (Mehta et al. 2016) was specific to uncultured Actinomycetes (Accession no. HQ388491). BLASTN (Altschul et al. 1990) was used to retrieve the homologous sequences (JN037867 and EU328054) from gene bank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). All three nucleotide sequences were aligned using program CLUSTALW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and were examined for the conserved regions. Specific nucleotide regions were selected to design the primers using Primer3 (v. 0.4.0) online tool (<http://frodo.wi.mit.edu/>).

Primer set	Name sequence (5' to 3')	Position	Bases	Homology (%)	Fragment size (bp)
PAC1F	AGCAAG TCGGCT GTGAAA GT	215–234	20	100	229
PAC1R	ACGTTT ACGGCG TGGACT AC	424–443	20	100	
PAC4F	TCGAGT CCGGTA GAGGAG AA	276–295	20	100	199
PAC4R	TCATTG GTATAA CCGCCA CA	455–474	20	100	

Position of each primer, homology and fragment size was obtained using nucleotide sequence HQ388491 (Mehta et al. 2016). To confirm the specific amplification by designed primers, a PCR analysis was performed with DNA from previously studied seven compost samples. 50 µl of PCR reaction mixture consisted of 5 µl 10X Taq Buffer, 1 µl of dNTPs (10 mM), 0.5 µl of each primer (10 µM), and 1.5 µl of Taq DNA polymerase (3 U/µl) (Genei, Bangalore, India) and 20 ng of compost DNA. The PCR cycle was as follows: 97 °C for 5 min, 35 cycles of denaturation at 97 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. All the PCR reactions were performed using gradient thermal cycler (Bio-rad, USA). The electrophoresis of PCR amplicon was done in 2% agarose gel, and to confirm the correct amplification it was cloned in pGEM-T (Promega, USA) and sequenced.

qRT-PCR analysis

Maxima SYBR Green/ROX qPCR master mix (Thermo Scientific, USA) was used with the Stratagene Mx Real-Time qPCR system (Stratagene, USA) for qRT-PCR. The specificity of primer set (PAC4F/PAC4R) was examined and the amplification of a specific transcript was confirmed by the appearance of a single peak in the melting curve analysis following completion of the amplification reaction. The relative level of specific bacterial population in compost DNA was normalized against the relative total bacterial population determined in each sample by using the 16S rRNA universal primers—Mf341 and Mr907 (Muyzer et al. 1995).

The reaction mixtures contained 2.5 ng of DNA, 1 µl of each primer (400 nM), 12.5 µl of Maxima SYBR Green/ROX qPCR master mix (Fermentas, USA) and PCR-grade water in a final volume of 25 µl. In all the experiments, appropriate negative controls containing no template were subjected to the same procedure to exclude or detect any possible contamination or carry-over. Each sample was amplified three times for each experiment and all the experiments were repeated at least twice. The program used included one step at 95 °C for 10 min, and 35 cycles consisting of 95 °C for 30 s, 58 °C for 30 s, and 72 for 40 s, followed by gradual heating (0.5 °C every 30 s) from 50 to 85 °C in order to generate the melting curve.

Results and discussion

Collection of composts and bioassay

Similar to our previous study (Mehta et al. 2016), a bioassay experiment was performed with seven new composts collected from nearby areas. After 15 days infection with pathogen, disease incidence was observed in the plants grown in pots mixed with compost. Fresh, dry biomass and percent disease in the plants were recorded. All composts were consistently suppressive to *Fusarium* wilt (Fig. 1). As compared to control plants, the most notably suppressive composts were Vermi Nagla (VN), Vermi Halduchaur (VH), Bamboo NADEP (BN), compost with *Trichoderma* (CT) and Vermi Khanna (VK) with no disease incidence. Composts Vermi Halduchaur Mature (VHM) and Raw NADEP were recorded with < 15% disease incidence. A significant difference was also observed in fresh biomass of the plants grown with compost mix. Maximum fresh biomass (2.80 g) was observed in the plants amended with CT while minimum (1.12 g) in VHM. Compared to control plants, a significant increase in dry biomass was also observed. Maximum dry biomass (0.188 g) was observed in the plants amended with VK while minimum (0.107 g) was recorded in VHM (excluding control plants).

Fig. 1 Percent disease, fresh and dry biomass of tomato plants grown in compost amended sand, infested with *Fusarium oxysporum* f. sp. *lycopersici*. Bars with the same letters are not statistically significant ($P \leq 0.05$). Details of the composts; VN Vermi Nagla, VH Vermi Halduchaur, VHM Vermi Halduchaur Mature, BN Bamboo NADEP, RN Raw NADEP, CT Compost with Trichoderma, VK Vermi Khanna

Validation of specific primer

To validate the specificity of designed primers, both set of primers (i.e., PAC1F/PAC1R and PAC4F/PAC4R) were tested on DNA isolated from previously studied compost (CPP: Cow Pit Pat-Supa; highly disease suppressive) and (JC: Jatropha cake + cow dung + *Cuscuta reflexa*; non-suppressive). After standardization of amplification condition only one set of primer (i.e., set PAC4F and PAC4R) resulted in successful amplification of 199 bp in highly disease-suppressive compost (i.e., CPP); however, no amplification was observed in non-suppressive compost (JC) (Fig. 2). For further confirmation, band amplicon was cloned and sequenced. Sequencing results confirmed its 100% similarity to the original sequence used in primer designing (HQ388491). After confirmation of specificity of designed primer for specific bacterial community, a similar experiment was repeated with new set of composts selected in present study. DNA was successfully isolated from all collected compost and PCR was performed with similar primer set. Amplification was observed in all selected composts except control (i.e., JC) and VHM. DNA template from all the composts was further tested for comparative study of population of specific bacterium by using real-time PCR analysis.

Real-time PCR for comparative study of population of specific uncultured bacterium in different composts

The primers designed to amplify specific population of uncultured bacterium resulted in a single melting peak in DNA extracted from all selected composts except in the compost with no disease suppression (JC). The comparative study of this specific bacterium in various composts was investigated by real-time-PCR using the primers PAC4F and PAC4R. The universal primers for 16S rRNA (Mf341 and Mr907) was used as internal control. The results showed that the population of specific bacterium

was detected in composts where there is disease suppression. Furthermore, this bacterium was differentially present in various composts and it is correlated with suppressive activity of that compost (Fig. 3a). In compost namely CPP, NADEP, Vermi Nagla, the population levels were higher than those in other composts (Fig. 3b). The growing evidence indicates that bacterium are involved in suppression of several soil borne diseases. Thus, we first time find out the presence of specific actinobacteria population in relation to *Fusarium* disease suppression in tomato seedlings by compost. Actinobacteria are very well known for degradation of lignin and in compost curing (Kirby 2005; Danon et al. 2008) and also for their antagonistic property against different plant and human pathogens (Cha et al. 2016; Fels et al. 2014). Most of the studies related to disease suppressiveness/antagonistic property are with cultured actinobacterium. However, there are a large number of uncultured actinobacteria which may have a great impact on the properties of compost. Present study also revealed the role of uncultured actinobacterium in disease suppression and confirmation of the specificity of this microbe by using molecular tools.

DNA-based markers are well studied and known for their specificity in different applications including genome mapping, gene tagging, genetic diversity, phylogenetic analysis and forensic investigations (Grover and Sharma 2016). Amplification with designed set of primer in disease suppressive composts reveals the specificity of PCR-based molecular marker. The high degree of similarity between the chosen community and amplified product implies that the primers are specific for selective actinobacterium community.

Since in the present study primers were designed to target specific microbial community of compost, accuracy of identifying selective community was very important. Results confirmed a clear relation between abundance of actinobacteria and disease suppressiveness property of compost.

Fig. 2 Semi-quantitative RT-PCR analysis with **a** seven previously studied composts and **b** seven new composts. “A” represents amplification with universal bacterial primers (Mf341 and Mr907) and “B” represents amplification with specific primers (Pac4f and Pac4r). “M” represents molecular marker. Compost details: *S-NADEP* Sak-

lani NADEP, *CP* cow dung + poultry waste, *JC* Jatropha cake + cow dung, *JCC* Jatropha cake + cow dung + *Cuscuta reflexa*, *JNMs* Jatropha cake + mushroom spent, *JNMsC* Jatropha cake + mushroom spent + *Cuscuta reflexa*, *CPP* Cow Pit Pat-Supa

Conclusion

Present study on the development of PCR-based molecular marker can be a valuable tool for preliminary testing of compost for its disease-suppressive nature confirmation to the results of bioassay test. This approach is particularly well

suitable to compost microorganisms that are difficult to identify or isolate because of the presence of other aggressive species. However, compost microbiology is still a complex phenomenon and there are possibilities of involvement of an individual microorganism or group of microorganisms in disease suppression. Therefore, in future there is a need

Fig. 3 The relative population of uncultured bacterium in various composts. **a** With pre-selected composts; **b** with new set of composts along with positive and negative control from previous study (highly disease suppressive; CPP and non-suppressive composts-JC)

to develop more molecular markers for rapid screening of compost.

Compliance with ethical standards

Conflict of interest The authors would like to declare that there is no conflict of interest with this research.

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