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Quantitative real-time PCR and high-resolution melting (HRM) analysis for strain-specific monitoring of fluorescent pseudomonads used as biocontrol agents against soil-borne pathogens of food crops

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ABSTRACT

Fluorescent pseudomonads colonizing roots of crop plants and producing antifungal metabolites are regarded as a reliable alternative to chemical fungicides against soil-borne phytopathogens. Key factors in successful pathogen control are presence and activity at the appropriate concentration, time, and place of biocontrol agents. Thus, quantification methods to monitor population dynamics are pivotal to the development of reliable application protocols. Real-time PCR is nowadays the most widespread culture-independent technique for the detection and enumeration of different target sequences. Here, its implementation with high resolution melting analysis as a powerful tool to accurately discriminate microbial inoculants is discussed.

1. Soil-borne pathogens and their biological control

Soil-borne plant pathogens, and particularly fungi and oomycetes, are among the most harmful pathogenic microorganisms in agricultural environments, representing a major limitation to the production of food crops worldwide (Raaijmakers, Paulitz, Steinberg, Alabouvette, & Moënne-Loccoz, 2009; Yadeta & Thomma, 2013). Examples of widely spread soil-borne fungi and oomycetes include *Fusarium* spp. (primarily *Fusarium oxysporum*), *Rhizoctonia solani, Gaeumannomyces graminis*, *Verticillium* spp. (mainly *V. dahliae*, *V. albo-atrum* and *V. longisporum*), *Phytophthora* spp. and *Pythium* spp.

Soil-borne pathogens are difficult to control: crop rotation, breeding for resistant plant varieties or genotypes, herbaceous grafting with resistant rootstocks, soil fumigations and application of pesticides are often insufficient to conveniently control root diseases. In past years methyl bromide, a very effective soil fumigant, was intensively used, but it has been banned since 2005 in all industrialized countries. The European Directive 91/414/EEC, and even more the following European Regulation 1107/2009 and European Directive 2009/128/EC, entailed a great re-evaluation of pesticides, that dramatically reduced the arsenal of available fumigants in European Countries (Colla, Gilardi, & Gullino, 2012), but also in other Countries exporting to Europe. The Directive also requires Member States to develop national action plans to further reduce the risk associated with the use of pesticides and promote the use of low-input systems (Matthews, Bateman, & Miller, 2014). The use of methods alternative to pesticides, such as biocontrol agents (BCAs), to control soil-borne pathogens had thus become increasingly relevant in the context of a general strategy that aims to reduce the environmental impact of agricultural practices (Colla et al., 2012). Besides, BCAs may provide control of diseases that cannot be easily managed by other control strategies, e.g. for cultivation in greenhouse, as reported by Paulitz and Bélanger (2001), or in hydroponics, as reported by Vallance et al. (2011).

Over the past fifty years, many research efforts have been made to screen for effective microorganisms to be used as antagonists against soil-borne pathogens. Some natural environments, known as suppressive soils, characterized by a very low level of disease development even when a virulent pathogen and susceptible host are present (Mazzola, 2002), occur worldwide (Haas & Défago, 2005). Natural suppressive soils are good examples of the indigenous microflora that effectively protects plants against soil-borne

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pathogens. The most important microorganisms involved in the natural suppressiveness of soil-borne plant pathogens and developed into BCAs are non-pathogenic fungi belonging to *Fusarium* spp. and *Trichoderma* spp., and bacteria belonging to *Pseudomonas* spp. and *Bacillus* spp. (Renault et al., 2007; Weller, Raaijmakers, McSpadden Gardener, & Thomashow, 2002).

2. Fluorescent pseudomonads

Fluorescent pseudomonads have been the most studied BCAs starting from 1970s (Thomashow, 1996; Weller, 2007). They are spread all over the world, exploiting many different ecological niches, from Arctic to tropical regions, and are particularly relevant in plant rhizosphere (Botelho & Mendonça-Hagler, 2006). It is well documented that biocontrol strains of fluorescent *Pseudomonas* can habitually colonize the root environment, representing up to 10% of all root-associated bacteria (Couillerot, Prigent-Combaret, Caballero-Mellado, & Moënne-Loccoz, 2009), and have the ability to protect plants from soil phytopathogens. As biocontrol agent, an organism must occupy an ecological niche similar to that of plant pathogens, and its mode of action must interfere both spatially and temporally with crucial steps in the development of pathogens (Paulitz & Bélanger, 2001). Fluorescent pseudomonads exert their beneficial effect via several different mechanisms, but principally by active exclusion of pathogens from rhizosphere (Rainey, 1999). More precisely, the recognized traits that make *Pseudomonas* spp. suitable for biocontrol are: competition with root pathogens for micronutrients (especially for iron and carbon) and aggressive root surface colonization (Haas & Défago, 2005; Raaijmakers et al., 2009); production of secondary metabolites that act as antimicrobial compounds, i.e. 2,4-diacetylphloroglucinol (DAPG), phenazines, pyrrolnitrin, pyoluteorin, hydrogen cyanide (HCN) (Handelsman & Stabb, 1996; Raaijmakers, Vlami, & de Souza, 2002); production of siderophores as pyoverdin, biosurfactants, extracellular lytic enzymes, effectors, cyclic lipopeptides (Raaijmakers, de Bruijn, & de Kock, 2006); stimulation of systemic reactions in plants, called rhizobacteria-mediated induced systemic resistance (ISR) (Bakker, Pieterse, & van Loon, 2007; van Loon, Bakker, & Pieterse, 1998).

Fluorescent pseudomonads demonstrated to be effective in many different pathosystems, i.e. to protect more than one plant species from often distinct pathogens, as long as the rhizosphere is successfully colonized (Couillerot et al., 2009; Haas & Défago, 2005). For example, strain CHA0 of *Pseudomonas protegens* (formerly, *Pseudomonas fluorescens*) (Ramette et al., 2011) isolated from roots of tobacco grown in soil suppressive to black root rot in Switzerland (Stutz, Défago, & Kern, 1986), demonstrated to be able to protect not only tobacco in different experiments, but likewise also cereals against *F. graminearum*, tomato against *F. oxysporum* f. sp. *radicis-lycopersici*, cucumber against *Pythium ultimum*, and peas against Fusarium wilt (Landa et al., 2002).

Similarly, *P. protegens* strain Pf-5, isolated from cotton rhizosphere and first described for its capacity to suppress seedling diseases of cotton caused by *R. solani* (Howell & Stipanovic, 1979) and *P. ultimum*, was demonstrated to suppress these pathogens also in cucumber, pea and maize, as well as *F. oxysporum* f. sp. *radicis-lycopersici* in tomato (Loper, Kobayashi, & Paulsen, 2007). *P. fluorescens* strain 2-79 was isolated in 1979 from the rhizosphere of wheat grown in a take-all disease suppressive soil in USA, and demonstrated to protect wheat against *G. graminis* (Weller & Cook, 1983), but also showed to protect other cereals (Couillerot et al., 2009). Moreover, *P. chlororaphis* strain PA23 was initially isolated from soybean root tips (Savchuk & Fernando, 2004), and both in greenhouse and field studies showed its ability to protect canola from stem rot caused by *Sclerotinia sclerotiorum*.

In conclusion, fluorescent pseudomonads have been mostly studied for protection of food crop plants from phytopathogenic oomycetes and fungi (*Pythium* spp., *F. oxysporum*, *G. graminis*, *R. solani*), and to a lesser extent for crop protection from bacteria (e.g. *Erwinia carotovorum*) and nematodes (e.g. *Meloidogyne* spp.) (Couillerot et al., 2009). They demonstrated to protect food crop plants from root pathogens also in hydroponics; for example, they proved to be effective against *Pythium aphanidermatum*, *P. ultimum* and *P. dissotocum*, in plants of cucumber and pepper growing in three different recirculating hydroponic systems (Pagliaccia, Ferrin, & Stanghellini, 2007).

Thanks to all the research efforts a number of *Pseudomonas* spp.-containing biocontrol inoculants have been commercially developed mainly in the US, and a few also in EU. The soil bacteria *P. chlororaphis* MA 342 is the active microorganism in Cedomon®, Cerall® and Cedress® (Lantmännen BioAgri AB, Sweden), *Pseudomonas* sp. strain DSMZ 13134 in Proradix® (Omya (Switzerland) AG Agro).

3. Real-time PCR: an established technology for detection and quantification of biocontrol agents including *Pseudomonas* spp.

The key aspect in biocontrol with *P. fluorescens* and closely-related species is to ensure their presence and activity at the appropriate concentration, time, and place. If a *Pseudomonas* strain is not able to compete with the microflora inhabiting the rhizosphere and to colonize the root surface, then it will not be an effective BCA. Thus, unambiguous strain identification among rhizobacteria and quantification methods to monitor population dynamics over time are pivotal to the development of reliable application protocols. Moreover, the registration process (Annex II of European Directive 2009/128/EC) for placing plant protection products based on microorganisms on the market establishes issues that need to be addressed, including the estimation of the fate and distribution of the microorganism in the environment and its side-effects on non-target species. These registration requirements in turn imply the use of monitoring methods that can accurately identify the released microorganism at strain level, and enabling its distinction from native strains of the same species, that are part of the microbial community of the rhizosphere.

Several methods can be used in field studies to assess the fate of the introduced beneficial bacteria (Ahmad, Husain, & Ahmad, 2011; Gamalero, Lingua, Berta, & Lemanceau, 2003). Methods for monitoring the released microorganisms can be classified in two major groups according to their reliance or not on cultivation *in vitro*, i.e. culture-dependent and culture-independent methods (Gamalero et al., 2003). The culture-dependent methods remain widely used mainly because they are easy to apply. However, these methods present several limitations: the detection of viable but not cultivable bacteria (VBNC) is not allowed, species/strain selective media are rarely available, the procedure is extremely time consuming, the results are not immediately accessible and not always conclusive since they do not allow discrimination of closely related organisms, and may require taxonomical skill for interpretation.

The culture-independent methods, especially those based on PCR are, conversely, easy to develop as species/strain specific, reliable, repeatable and quick. Since conventional PCR easily allows the detection of a microorganism, but not its precise quantification, real-time PCR (or q-PCR) has become nowadays the most widespread culture-independent technique to quantify target sequences (Sanzani, Li Destri Nicosia, Faedda, Cacciola, & Schena, 2014; Sørensen, Nicolaisen, Ron, & Simonet, 2009). Several real-time PCR approaches have recently been developed for the detection and enumeration of different biocontrol agents, including

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fluorescent *Pseudomonas* spp. (El Hamouchi et al., 2008; von Felten, Défago, & Maurhofer, 2010; Savazzini, Oliveira Longa, Pertot, & Gessler, 2008).

In real-time PCR the amplicons are measured at an early stage of the reaction when the efficiency is still constant. The number of PCR cycles necessary to generate a fluorescent signal significantly above the noise level is inversely related to the log of the initial amount of target molecules. Quantification is automatically determined by interpolating cycle threshold (Ct) values of unknown samples with standard curves prepared from known quantities of the target DNA (Fig. 1A and B). Standard curves are also useful to determine the linear dynamic range and the efficiency of the reaction which should be as closed as possible to 100% (Bustin et al., 2009).

The efficiency of PCR reaction can be influenced by non-target host plant DNA and particularly by the presence of inhibitors (compounds of different chemical nature, i.e. polyphenols and polysaccharides) in the plant DNA extracts. Hence, in the case of real-time PCRs having rhizobacteria biocontrol agents as target, the use of a solution of total genomic DNA extracted from roots of untreated plants is recommended for the preparation of serial dilutions of a plasmid DNA containing the target of interest. In this way, the standard curve is established with samples of known target DNA quantities resembling as much as possible to natural samples. Moreover, it has been suggested to normalize quantification data of the target DNA with quantification data of host DNA in order to avoid interference of sample size and extraction efficiency from one sample to another (van Gent-Pelzer, Krijger, & Bonants, 2010).

Several chemistry formats have been developed for the real-time PCR assay; the reader is referred to Schena et al. (2013) for a detailed treatise. The most frequently used among the specific and non-specific methods are TaqMan® probes and SYBR® Green I, respectively. The results of recent researches, however, provided a new generation of intercalating dyes such as SYTO® 9, EvaGreen® and LCGreen®. As it is detailed below, these newly introduced dyes allowed a finer monitoring of intercalation dynamics, thus expanding the range of application of q-PCR. Among them, EvaGreen® seems very promising, exhibiting many advantages as compared with the most used intercalating dye SYBR® Green I. Mao, Leung, and Xin (2007) and Eischeid (2011) demonstrated for EvaGreen® a higher reproducibility, a lower PCR inhibition effect, a weaker binding for short dsDNA fragments (which reflects the low tendency to promote not specific amplification), as well as higher and narrowed melting curves compared with SYBR® Green I.

3.1. *Development of real-time PCR markers for species/strain identification and quantification of bacterial biocontrol agents including Pseudomonas spp.*

A crucial step in the development of a real-time PCR assay is the identification of appropriate target DNA regions. It is now widely accepted that a good target gene should readily be amplified and sequenced, and has to be present in single-copy in the genome in order to most accurately correlate Ct values with the amount of the microorganism cells. Real-time PCR markers for detection and identification of bacterial microorganisms at species or even at strain level have been developed through two main strategies: 1) from genes or sequences with known functions such as the 'housekeeping' protein coding genes, or 2) from regions of the microorganism's genome with unknown functions such as the sequence-characterized amplified region (SCAR) markers (Schena et al., 2013).

In the first strategy, the same known conserved gene is amplified and sequenced from target and non-target microorganisms with universal primers. Regions of the sequence that are different are used to design primers for PCR.

The most commonly used target for bacteria is the DNA encoding the 16S ribosomal RNA gene, since it is highly stable, possesses conserved as well as variable sequences, and can be amplified and sequenced with universal primers. However, the capability of this

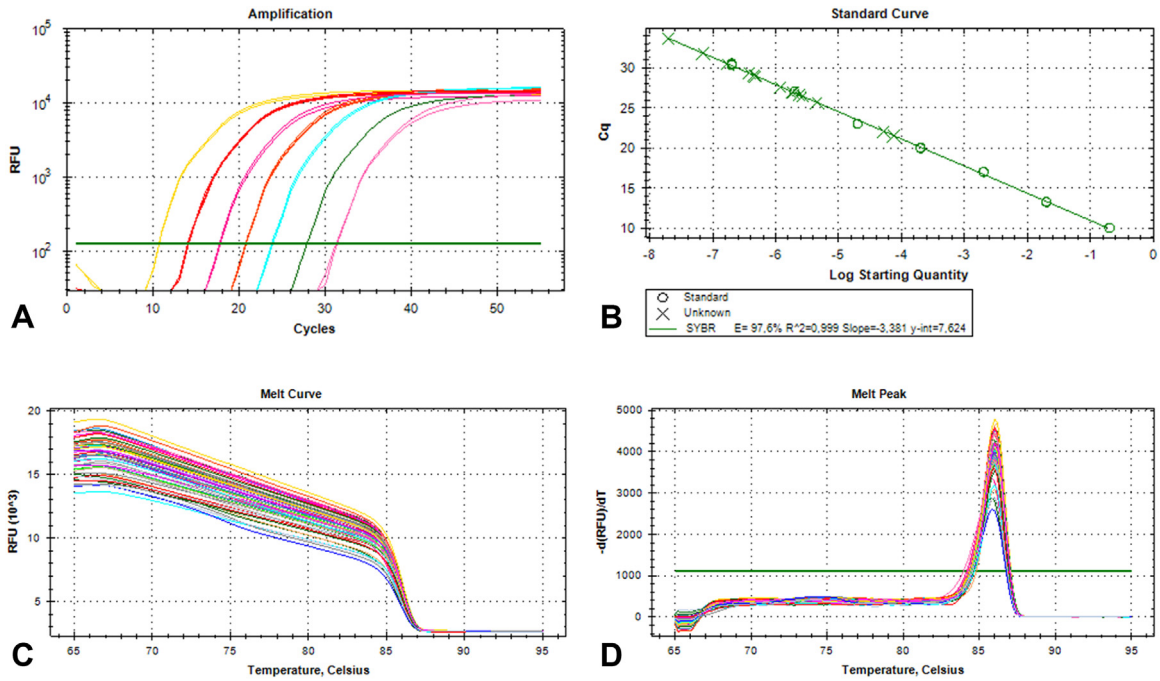

Fig. 1. Amplification curves of 1:10 serial dilutions of a plasmid containing *gyrB* gene of *P. protegens* Pf4 (A), standard curve established for the Pf4 quantitation in DNA samples extracted from Pf4-treated and untreated roots (B); melting curves (C) and melting peaks (D) of amplicons generated from DNA samples extracted from Pf4-treated and untreated roots.

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region to differentiate closely related taxa is quite limited because of its extremely slow rate of evolution. Therefore, alternative targets for designing primers, more variable than 16S rRNA gene and able to discriminate closely related taxa, have been investigated.

Within fluorescent *Pseudomonas* spp., in addition to 16S rRNA gene, the antifungal metabolite-encoding genes *phlD* (a key gene in the biosynthesis of 2,4-DAPG) (De La Fuente, Mavrodi, Landa, Thomashow, & Weller, 2006) and *hcnBC* (hydrogen cyanide synthesis gene) (Ramette, Moënne-Loccoz, & Défago, 2006), and *gacA* (response regulator gene) (De Souza, Mazzola, & Raaijmakers, 2003) gene have been used as markers of genetic diversity, suggesting that they may be used as targets for fluorescent *Pseudomonas* species or strain-specific real-time PCR assays. Within *Pseudomonas* spp., other eligible targets are the 'housekeeping' protein coding genes *gyrB* (beta-subunit of gyrase), *rpoD* (sigma 70 subunit of RNA polymerase) and *rpoB* (beta-subunit of RNA polymerase), which are currently proposed besides 16S rRNA gene in a MLSA approach for studying the phylogeny of the genus *Pseudomonas*, as well as for ascribing novel strains to known species (Gomila, Peña, Mulet, Lalucat, & García-Valdés, 2015; Mulet, Bennasar, Lalucat, & García-Valdés, 2009; Mulet, Lalucat, & García-Valdés, 2010).

In the second strategy, specific target sequences have been identified by amplifying random regions of the bacterial genome with PCR-based techniques, such as random amplified polymorphic DNA (RAPD), arbitrarily primed PCR (AP-PCR) and amplified fragment length polymorphism (AFLP). Recently, microsatellite primed-PCR (MP-PCR) and repetitive PCR (rep-PCR) have been regarded as more robust than RAPDs, because longer primers are used for MP-PCR and rep-PCR as compared to RAPDs (Ma & Michailides, 2007). This allows more stringent annealing temperatures and reaction conditions that enhance reproducibility. This approach requires more time and experience compared to the amplification of conserved genes, since the analysis of a large number of isolates of closely related taxa is necessary. However, SCAR primers have been shown to be particularly useful when other target genes do not allow to differentiate closely related taxa or when specific strains need to be identified as in the case of biocontrol agents, e.g. *P. protegens* CHAO (von Felten et al., 2010) and *Pantoea agglomerans* CPA-2 (Soto-Muñoz, Teixidó, Usall, Viñas, & Torres, 2014).

3.2. Real-time PCR methods developed for Pseudomonas spp.

Several real-time PCR approaches have recently been developed for the detection and enumeration of different *Pseudomonas* spp. biocontrol agents; examples of such methods are given in Table 1. Checking on the chemistry formats which have been developed for these real-time PCR assays, it appears that dsDNA-intercalating dyes have been extensively used, especially SYBR® Green I (von Felten et al., 2010; Holmberg, Melin, Levenfors, & Sundh, 2009; Mavrodi, Mavrodi, Thomashow, & Weller, 2007) but also the dsDNA-intercalating dye of new generation EvaGreen® (Moruzzi et al., submitted) (Fig. 1). These findings confirm that a fully optimized real-time assay based on dsDNA-intercalating dye can be used for detection and quantification of the target molecule as a simple and reliable low-cost method. However, *Pseudomonas*-based BCAs have also been monitored with the TaqMan probe, although to a more limited extent, to assess for example the environmental fate of *P. fluorescens* strain EPS62e after its introduction into apple phyllosphere against fire blight (Pujol, Badosa, Manceau, & Montesinos, 2006).

Moreover, both strategies concerning the PCR markers (i.e. primers development from genes with known function and from SCAR markers) have been used and regarded as attractive for developing *Pseudomonas* spp. strain-specific real-time PCR primers.

Genes with known function chosen as real-time PCR targets were *phlD* gene for the detection of four different genotypes of *phlD*$^+$ *P. fluorescens* strains (Mavrodi et al., 2007) and *gyrB* gene for the detection of *P. protegens* Pf4 potential biocontrol agent against root rot caused by *R. solani* (Moruzzi et al., submitted).

SCAR markers have been developed for the monitoring of *P. fluorescens* EPS62e in the apple phyllosphere (Pujol et al., 2006), but also for the assessment of several *Pseudomonas*-based BCAs fate in the rhizosphere of maize as in the case of strain-specific quantification of the three biocontrol fluorescent *Pseudomonas* strains F113, CHAO and Pf153 (von Felten et al., 2010). Furthermore, SCAR markers were used to develop q-PCR assays for the monitoring of the biocontrol candidate strain *Pseudomonas brassicacearum* MA250 (effective against snow mould, *Microdochium nivale*) on different parts of wheat seedlings (Holmberg et al., 2009).

The detection limits of the optimized real-time PCR assays included in Table 1 were similar, corresponding to about 10 CFU with DNA extracted from pure cultures and to 100–1000 CFU for bacterial DNA extracted from spiked environmental samples, suggesting that similar performances can be reached regardless of the chemistry and target chosen for the quantitative PCR assays. Furthermore, the detection limit is below the minimal threshold BCA population size (10^5–10^6 CFU/g root), which is required for a successful control of soil-borne pathogens in the rhizosphere (Haas & Défago, 2005).

In the listed manuscripts the persistence and concentration of BCAs after their application in the environment, were monitored also by conventional culturing methods besides the quantitative molecular methods. From comparison of results obtained with the two methods, it is clear that in general DNA-based quantification methods detect a higher BCA population size than culture-based methods. This difference is usually attributed to the fact that DNA-based methods also detect dead cells or nonculturable but viable cells (Mavrodi et al., 2007; Rezzonico, Moënne-Loccoz, & Défago, 2003).

Table 1
Quantitative real-time PCR methods developed for monitoring *Pseudomonas* spp. biocontrol strains in the environment.

Bacterial strain	PCR marker	Chemistry format	Reference
Pseudomonas fluorescens EPS62e	SCAR	TaqMan® probe	Pujol et al., 2006
Pseudomonas protegens Pf-5	*phlD*	SYBR® Green I	Mavrodi et al., 2007
Pseudomonas fluorescens Q2-87	*phlD*	SYBR® Green I	Mavrodi et al., 2007
Pseudomonas fluorescens Q8r1-96 and FTAD1R34	*phlD*	SYBR® Green I	Mavrodi et al., 2007
Pseudomonas fluorescens FTAD1R36	*phlD*	SYBR® Green I	Mavrodi et al., 2007
Pseudomonas brassicacearum MA250	SCAR	SYBR® Green I	Holmberg et al., 2009
Pseudomonas fluorescens F113	SCAR	SYBR® Green I	von Felten et al., 2010
Pseudomonas protegens CHAO	SCAR	SYBR® Green I	von Felten et al., 2010
Pseudomonas fluorescens Pf153	SCAR	SYBR® Green I	von Felten et al., 2010
Pseudomonas protegens Pf4	*gyrB*	EvaGreen®	Moruzzi et al., submitted

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4. Implementation with high-resolution melting (HRM) analysis, as an innovative molecular approach in the diagnostics of bacteria inoculants

As stated above, the introduction of the latest generation dyes allowed technical strategies in q-PCR. A particularly useful and interesting approach to the identification of microorganisms at species and strain level is the so-called high resolution melting (HRM) analysis, an advanced method based on melting behaviour of double stranded DNA (Tong & Giffard, 2012).

4.1. Principles of high resolution melting (HRM) analysis and data analysis

High resolution melting (HRM) analysis is an automated analytical molecular technique which measures the rate of double stranded DNA dissociation to single stranded DNA with increasing temperature (Reed & Wittwer, 2004). The temperature at which 50% of the DNA is dissociated is called melting temperature (Tm), which depends both on the length and the percent guanidine-cytosine (GC) content of the DNA fragment. As mentioned above, melting curve analysis is frequently used in real-time PCR to check if the correct amplicons have been generated (low-resolution melting).

A requirement for performing a melting curve analysis is the incorporation of dsDNA binding dye in the amplified DNA fragment. However the requirements of the dyes used for HRM are different from dyes normally used for standard quantitative real-time PCR assays. In fact, HRM takes advantage of third generation intercalating dyes such as EvaGreen®, LCGreen® and SYTO 9®. These dyes are used at higher concentration for greater saturation of dsDNA and less redistribution from the melted regions of single-stranded DNA back to the regions of dsDNA (Reed, Kent, & Wittwer, 2007). Dyes such as SYTO 9® and LCGreen® are saturating dyes ensuring more complete intercalation of the amplicon, without inhibition of DNA polymerases or modification of the Tm of the product. Dyes such as EvaGreen®, which is a "release-on-demand" dye, can be added at non-saturating concentrations, thus ensuring no PCR inhibition, with their fluorescence quenched as long as they are not bound to DNA. Upon binding to dsDNA, the quenching factors are released and the dyes emit high fluorescent signal.

Besides third generation dyes, another requirement of HRM is high-resolution instruments. Since it is convenient to have both functions of amplification and melting analysis combined in one instrument, several real-time PCR thermal cyclers have been adapted to high-resolution melting analysis. After the step of real-time PCR, melting of the amplicons is carried out by gradually increasing the temperature with smaller temperature increments (0.01–0.2 °C) than low-resolution melting analysis. During melting of the PCR product, the intercalated dye is released. HRM analysis scans the entire melting process; thus, the generated melting profile is based on all temperature points rather than peak points as in low-resolution melting analysis. Moreover the dye labels the PCR product along its entire length in order to detect efficiently all melting domains (Reed et al., 2007).

With suitable software, all the data of relative fluorescence intensity collected during the melting process are plotted against the temperature to generate raw melt curve data (Fig. 2A). The software proceeds with the normalization of relative fluorescence intensity so that the average data value at the start of the pre-melt region is 1, and at the end of the post-melt region is 0 (Fig. 2B). Differences in melting curve shapes can be analysed generating "Difference Curves", subtracting the sample melting curve from the melting curve of a reference sample (Fig. 2C). Therefore, samples with similar melting curves are clustered automatically into groups. The identity of samples can be verified by comparing the melting curves with those of reference strains. Reliability of the clusters can be evaluated by the software calculating confidence values, thus reducing subjectivity in the interpretation of the results.

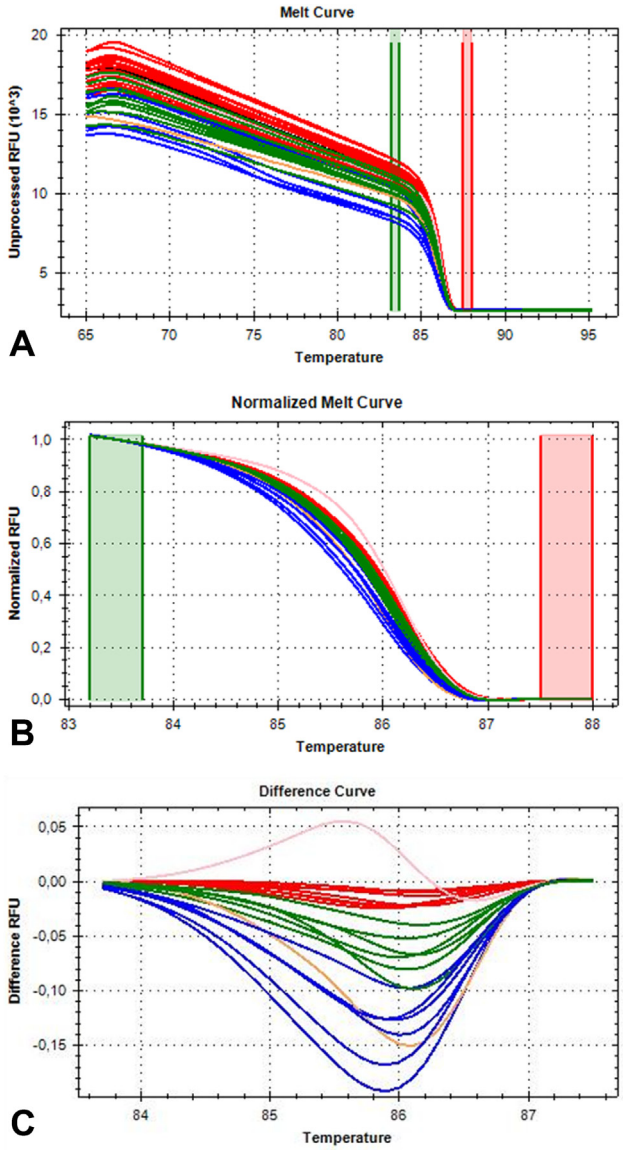

Fig. 2. Raw melt curve data (A), normalized melt curve (B) and difference curves in relation to control sample (red curve) (C) generated from HRM analysis on amplicons obtained from DNA of root samples collected from *P. protegens* Pf4-treated or untreated plants. All the Pf4-treated samples formed a unique red cluster together with the positive control represented by Pf4 total genomic DNA, whereas untreated samples formed four different clusters indicated with green, blue, pink and orange curves.(For interpretation of the references to colour in this figure caption, the reader is referred to the web version of this article.)

There are several important aspects that should be taken into consideration in order to develop a successful HRM analysis method. When designing primers special attention should be paid on preventing amplification of non-specific products and formation of primer-dimers. Moreover, amplicon length should not exceed 300 base pair (bp), since the length of the amplicon influences the sensitivity of following HRM analysis, and the secondary structure within the amplified product should be investigated because it may result in unusual melting profiles. Finally, the PCR conditions have

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to be optimised in order to increase efficiency of amplification, indicated by lower Ct values and amplification curves reaching the plateau phase. Volume of reaction should be the same for all samples, with the same concentration of dye and of buffer, Mg^{2+} and other salts which can affect the DNA melting behaviour. Especially $MgCl_2$ concentration is one of the parameters which more strongly influences the melting behaviour of dsDNA, therefore particular attention should be paid to its optimization. All DNA samples to be analysed should be extracted using the same DNA extraction method or kit, and should be of high integrity and purity; moreover, the amount of DNA added to the reaction mixture should not differ significantly between samples in order to get similar Ct values (Reed et al., 2007). Samples with very low DNA template producing Ct values higher than 30, or samples generating curves not sigmoid or near sigmoid should be discarded from the analysis.

4.2. Applications of HRM analysis including diagnostics of bacteria inoculants

HRM is an incredibly sensitive and accurate technique, useful to rapidly identify DNA sequence variants, SNPs and mutations, therefore it plays an important role in clinical research and diagnostics. In the last few years this novel DNA-based method has been investigated and successively applied in many different research areas, such as diagnostics of parasites and pathogens in humans (Druml & Cichna-Markl, 2014; Tong & Giffard, 2012) and animals (Ghorashi, Noormohammadi, & Markham, 2010), detection and analysis of cancer-related mutations in humans (Simi et al., 2008), plant genotyping (Mackay, Wright, & Bonfiglioli, 2008), authentication of plant and food products (Druml & Cichna-Markl, 2014; Ganopoulos, Argiriou, & Tsaftaris, 2011).

Focussing on human pathogens and food safety aspects, a number of papers have shown that HRM analysis can be applied for genotyping and serotyping of foodborne related pathogenic microorganisms such as *Listeria* spp, *Salmonella* spp., *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus* (Druml & Cichna-Markl, 2014) with very high discriminatory power. For instance, Lilliebridge, Tong, Giffard, and Holt (2011) applied HRM for genotyping of *S. aureus* using six different DNA fragment containing SNPs as target. The HRM analysis on 94 isolates of *S. aureus* yielded 268 melting types. Kagkli, Folloni, Barbau-Piednoir, Van den Eede, and Van den Bulcke (2012) developed HRM analysis targeting *stx1* (Shiga toxin 1), *stx2* (Shiga toxin 2) and *eae* (virulence facto intimin) genes for differentiation of *E. coli* strains. The authors were able to discriminate between strains based on the toxin variant they possess. Furthermore, they showed that also multiplex PCR can be coupled with HRM analysis.

In the plant pathology research field, HRM has been inferred in relatively few studies to discriminate pathogenic microorganisms such as viruses (Bester, Jooste, Maree, & Burger, 2012), bacteria (Gori, Cerboneschi, & Tegli, 2012) and fungi (Ganopoulos, Madesis, Zambounis, & Tsaftaris, 2012; Zambounis, Ganopoulos, Chatzidimopoulos, Tsaftaris, & Madesis, 2014).

Regarding bacteria plant pathogens a unique strategy based on real-time PCR followed by HRM analysis was reported for the rapid, highly specific and sensitive detection and identification of three different *Pseudomonas savastanoi* pathovars, namely *P. savastanoi* pv. savastanoi (*Psv*), pv. nerii (*Psn*) and pv. fraxini (*Psf*), respectively (Gori et al., 2012). The HRM analysis-based assay allowed to unequivocally discriminate *Psv*, *Psn* and *Psf* according to several single nucleotide polymorphisms found in their Type Three Secretion System clusters. In addition, Gori et al. (2012) demonstrated the feasibility of developing a multiplex-HRM protocol.

In the research area of biological control, recently we obtained the first results concerning the application of HRM analysis in diagnostics of a beneficial bacterial strain of *P. protegens* Pf4 against soil-borne pathogens with the aim to easily discriminate the strain of bacterial inoculant from those strains of the same species naturally residing in the soil at the rhizosphere level (indigenous microflora) (Moruzzi et al., submitted). As an example of development and application of a molecular tool for specific detection and quantification of a *Pseudomonas*-based BCA, the steps involved in the research work conducted within the frame of AGER Stay-Fresh project, are summarized in Fig. 3. This cultivation-independent method, based on a strain-specific real-time PCR assay followed by HRM analysis, has been developed on *gyrB* gene (Table 1) (Moruzzi et al., submitted), which resulted to be the most appropriate target to be used for the development of a Pf4 strain-specific assay, compared to *rpoD* (sigma 70 subunit of RNA polymerase) and *secY* (preprotein translocase membrane subunit) genes. Primers and product size were determined following the suggestions given above.

After optimization of the real-time PCR assay, this molecular method was used to accurately monitor the presence and quantity of Pf4 bacterial cells during the *in vivo* test (Fig. 2), which included Pf4-treatments on lamb's lettuce against *R. solani* root rot in small-scale hydroponics aimed to determine its disease suppressiveness. The treatment with Pf4 reduced of about 40% the percentage of wilted plants (Fig. 4) indicating an actual protective effect of Pf4 against *R. solani* root rot. Quantification of Pf4 with strain-specific real-time PCR assay demonstrated that the density of Pf4 above the threshold value of 10^5 CFU/g of root required for suppression of root diseases (Haas & Défago, 2005), was maintained for the whole lamb's lettuce growing period.

5. Future perspectives

Molecular tools offer new possibilities to enhance research on biocontrol agents. Comprehension of the population dynamics of BCAs is decisive for predicting the success of BCAs in disease control. The *Pseudomonas* strains used as BCAs must compete within the environment of the rhizosphere, so it is crucial that they reach a fitting concentration (the threshold value of 10^5 CFU/g of root), in the right time and place.

Real-time PCR had become a standard technique for detection and quantification of microorganisms in many laboratories, including those involved in biological control research. Despite its age, PCR-based technology is still in rapid evolution. The well-known advantages of real-time PCR over conventional detection and quantification methods (i.e. possibility to avoid microorganism culturing, high sensitivity, versatility, rapidity, accuracy and reliability, and high-throughput DNA detection and quantification from various environmental samples) are now being complemented with the recent improvements provided by the introduction of intercalating dyes of new generation. Current applications of EvaGreen® include quantitative PCR, HRM analysis, real-time isothermal DNA amplifications and capillary gel electrophoresis.

Exploiting the performances of the EvaGreen®-based novel chemistry, HRM represents a significant advance over conventional melting curve analysis; contrary to low resolution melting, it allows the precise definition of the melting profile of an amplicon. Since the melting profile depends mostly on base composition and size of the amplicon, HRM is highly suitable for detection of SNPs and small insertions or deletions; therefore, it has the potential to finely discriminate the microorganism of interest from those closely-related present within a plant or in the environment.

We envisage that other biocontrol researches involving the application of potential BCA in the environment would benefit from this molecular approach, which has several advantages over

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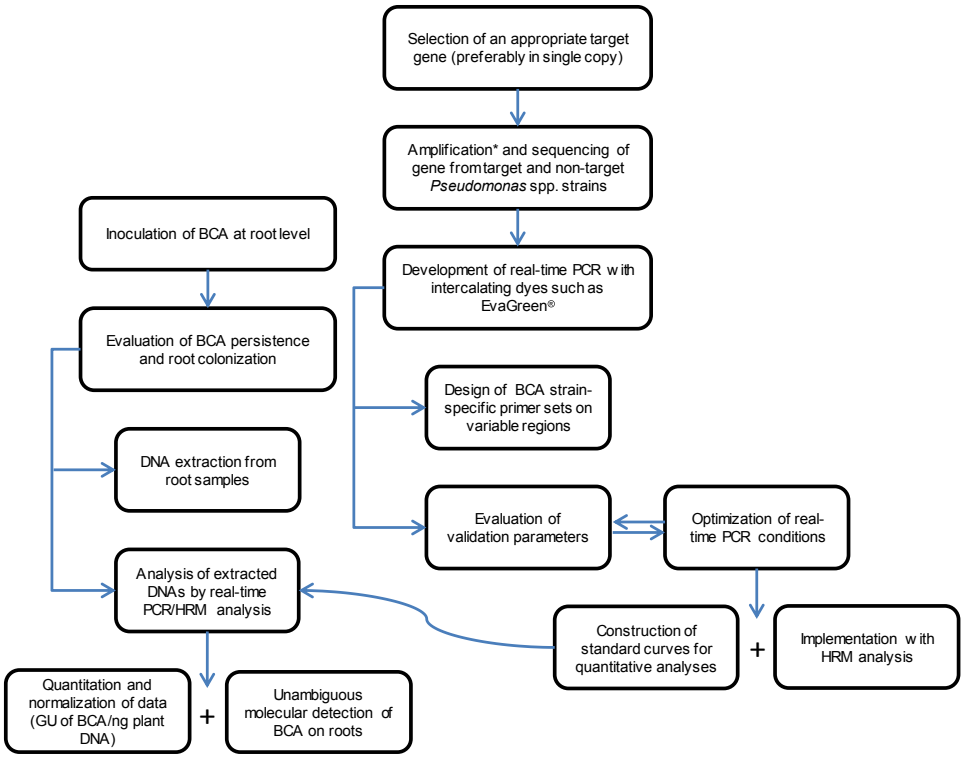

Fig. 3. Schematic flowchart of the steps which can be followed for the development of real-time PCR/HRM assays specific for *Pseudomonas*-based BCA strains to be used for their monitoring in *in vivo* tests. The same or similar flowchart can be adopted for potential BCAs, other than *Pseudomonas* spp. strains. (*: amplification with de novo primers or primers published in the literature).

traditional methods for genotyping: it is a high-throughput method, it is faster and less laborious since it does not require manual post-PCR processing; in addition, the entire process is performed in a close-tube, thus the risk of contamination is greatly reduced. HRM is also less expensive if compared to alternative approaches based on the use of probes or sequencing.

Despite the broad application of the DNA-based quantification methods, the inability of differentiate between viable and non-viable cells, still represents a major challenge for scientists. The degradation of DNA after cell death seems to strongly depend on environmental conditions; there are reports showing a quick degradation of DNA, whereas others demonstrate a long persistence of it with the consequent overestimation of microbial density. However, to overcome this problem, a promising strategy relies on the introduction of a sample pre-treatment with an intercalating dye, such as propidium monoazide (PMA), before real-time PCR analysis. PMA intercalates DNA from dead cells with compromised cell membranes, upon visible light exposure it binds covalently to DNA thus inhibiting PCR reaction. PMA treatment combined with real-time PCR has been successively used for the quantification of viable cells of foodborne pathogenic microorganisms such as *Listeria monocytogenes* (Pan & Breidt, 2007), *E. coli* 0157:H7 (Elizaquível, Sánchez, Selma, & Aznar, 2012) and *Campylobacter jejuni* (Josefsen et al., 2010), but also for viable cells of the biocontrol agent *Pantoea agglomerans* CPA-2, effective against the major postharvest diseases of pome and citrus fruits (Soto-Muñoz et al., 2014). Thus, in the future it will be interesting to verify the feasibility of the introduction of a pre-treatment with PMA to *Pseudomonas*-based BCAs before real-time PCR quantification.

Fig. 4. Symptoms of wilting on untreated (A) and *P. protegens* Pf4-treated (B) lamb's lettuce plants, 14 days after artificial infection with the soil-borne fungal pathogen *R. solani*.

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