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Identification and quantification of viruliferous and non-viruliferous *Polymyxa betae*

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Abstract

Rhizomania, caused by Beet Necrotic Yellow Vein Virus (BNYVV) is transmitted by plasmodiophorid *Polymyxa betae*. To investigate quantification of virulifeous and non- viruliferous *P. betae* isolates, different techniques including serological method (DAS- ELISA), PCR- based method and nanobiocensor method have been used. For this purpose, sugar beet susceptible cultivar (Regina) was cultivated in soils of different regions in greenhouse conditions. Six weeks after planting, lateral roots of beets from each soil were visually tested through microscopy and the of *P. betae* cystosori was seen and the lateral root sap was prepared. Then DAS- ELISA with polyclonal antibody against recombinant expressed fungal glutathione-s- transferase isolates of Shiraz was optimized. Optical density of different samples were calculated for both the vector and the virus using ELISA method. Simultaneously, confirmation of quantitative estimation *P. betae* in lateral root was conducted by nanobiosensor against vector. Nanobiosensor method was performed based on Florescent Resonance Transfer Energy (FRET) using antibody attached quantom dots and GST conjugated rhodamine. Microscopic results show presence of vector in all soils. BNYVV was found in soils Fars, Khorasan, Hamadan and Kermanshah. In soils of Azarbayjan, Gorgan, Dezfool, Kerman, Karaj and Arak were found no virus. Values of optical density of *P. betae* in soils with and without virus have no significantly difference. Because of high speed and sensitivity of nanobiosensor, its use for quantitative estimation of *P. betae* has been advised.

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Introduction

The protist *Polymyxa betae* Keskin is an obligate parasite of sugarbeet roots and the plasmodiophorid vector of Beet Necrotic Yellow Vein Virus (BNYVV), which causes rhizomania disease. *P. betae* is found in almost all soils where sugarbeet is grown, spreading from plant to plant by means of motile zoospores and survive in the soil for many years in the form of thicked-wall resting spores or cystosori (Rush, 2003). Despite its ubiquitous distribution and parasitic habitat, *P. betae* is generally considered to cause relatively little damage in temperate climates, although it may be pathogenic in areas of the world where sugarbeet is grown in warm soils (Blunt *et al*., 1991). In contrast, rhizomania disease causes severe economic losses in many countries and is spreading into new regions (McGrann *et al*, 2009). In Iran, it was reported from the Fars province in 1996 and is now found in nearly all sugarbeet-growing areas of the country (Izadpanah *et al*., 1996; Sohi and Maleki 2004). *P. betae*, the sole vector of BNYVV, has attracted increasing attention in recent years in Iran, because its distribution and behavior determine the incidence and severity of the disease. However, because it is an obligate parasite, epidemiological studies, and the search for potential sources of host resistance to *P. betae*, have required bioassays procedures, the evaluation of which can only be achieved by lengthy and laborious microscopic examination of roots (Mutassa-Gottgens *et al*., 2000).

Traditional methods to detect and quantify vector and virus in soil are based on bait plant bioassays using soil dilutions to estimate the most probable numbers (MPN) of infective propagules (Tuitert, 1990). These methods are expensive and time-consuming, taking more than 8 weeks to complete for a single soil sample. There was a need to develop a rapid, accurate and specific detection and quantification method for the *P. betae* in roots. DNA-based tests were developed which were able to identify the presence or absence of *P. betae* within the plan, but unable to quantify the relative amounts of the pathogen. Another limitation of DNA-based tests is that they cannot determine if the parasite is alive or dead (Kingsnorth *et al*., 2000). Serological tests that recognize proteins, which can be less stable than DNA, may also be able to distinguish between viable and nonviable cells. Using ELISA as a detection method has the main advantage that amounts of protein can be quantified. Also, it is relatively quick and easy, without the need for expensive laboratory equipment, and it can be automated for rapid on-line testing. Polyclonal antibodies have been used in ELISA tests for *Spongospora subterranea* (Merz *et al*., 2005), *P. betae* (Mutassa-Gottgens *et al*., 2000 and Kingsnorth *et al*., 2003a), *Polymyxa. graminis* (Delfosse *et al*., 2000) and *Plasmodiophora brassica* (Wakeham and White 1996). All authors reported a (semi-) quantitative detection of resting spores in plant material and soil samples.

Glutathione-S-transferase (GST), a specific immunogenic protein, is a critical enzyme expressed in *P. betae*`s zoospores, sporangia and resting spores and could be regarded as a good candidate for the development of the biobase of antibody and nanobiosensor. In fact, the pathogen expresses GST at high levels to overcome host defense mechanisms (Mutasa *et al.,* 2000). Antibody to *P. betae* has been developed in Iran recently (Safarpour *et al*., 2012a) and is widely available for quantitative detection of it. One of the most important nanomaterials is fluorescent semiconductor nanocrystals, also known as quantum dots (QDs) which have been widely used for disease diagnosis (Frasco and Chaniotakis, 2009). QDs have a number of unique optical properties that are advantageous in the development of bio-analyses based on fluorescence resonance energy transfer (FRET) (Algar and Krull, 2007). QDs have been reportedly used as biosensors by coating them with specific antibodies against various pathogenic agents such as *E. coli* O157:H7 (Hahn *et al.,* 2008). Moreover, a quantum dots FRET-based nano-biosensor for efficient detection of *P. betae* was developed in Iran (Safarpour *et al*., 2012b). The purpose of this study was to identify and quantify viruliferous and nonviruliferous *P. betae* isolates in different sugarbeet cultivation of Iran firstly using

serological and nanobiosensore methods that recently were developed in Iran and PCR- based method.

Material and methods

Soil sampling and bait plant technique

Soil samples were taken from fields where sugarbeet is really grown, including the fields with rhizomania occurrence in the past and with no problems concerning sugarbeet growing. Altogether, about 6 kilograms of soil was taken on several sites from each field. Then, soil samples were dried at room temperature in a laboratory and sieved through 2 mm screens. Sugarbeet baiting plants (cv. Regina) were sown into pots with soil samples mixed in equal parts with autoclaved compost to facilitate ease of root removal of bait plants at harvest. The plants were grown under controlled conditions with a 16-h photoperiod at 20°C (night) and 23°C (day). The pots were watered directly as needed. After six weeks, roots of baiting plants were harvested, washed and examined by optical microscope for the presence of *P. betae*. If they were found, roots were tested for the presence of BNYVV by enzyme linked immunosorbent assay (ELISA).

Molecular identification of P. betae

In molecular method, presence of *P. betae* in roots of plants was detected with amplification of rDNA of plasmodiophoromycetes and *P. betae* species, simultaneously. The DNA extraction from sugar beet lateral roots was done according to Mutasa-Gottgens *et al.* (2000). Tow primer pairs including: Psp1 5´-TAGACGCAGGTCATCAACCT-3´, Psp2rev 5´-AGGGCTCTCGAAAGCGCAA-3´ and PB4 5´-CAAAGGCCTGAAATCATCTAAC-3´, PB4rev:5´-GATGGCCCAATTCCTTACAC-3´ were used to amplify a 454 and 170 bp region of the nuclear ribosomal DNA of *P. betae* and repetitive *Eco*RI like region, respectively (Legrève *et al.* 2003; Munier *et al.*, 2003). The following PCR program was conducted: 95 °C for 2 min, 36 cycles of 96 °C for 45 s, 50 °C for 45 s and 72 °Cfor 1 min and final synthesis for 10 min 72 °C. Sugarbeet roots free of *P. betae* were used as negative control.

BNYVV/P. betae ELISA detection and quantification

The roots of sugarbeet plants were tested for the presence of *P. betae* and BNYVV by ELISA test. The double-antibody sandwich (DAS)-ELISA was optimized to determine *P. betae* infection by using the specific polyclonal antibody against GST protein of *P. betae* (kindly provided by H. Safarpour, ABRII, Karaj, Iran). Also, a DAS-ELISA was used for detection of BNYVV infection in the same lateral roots using antibody supplied by Plant Virology Research Center (Shiraz, Iran). All these tests were performed as described by Clark & Adams (1977) with modifications. The plates were measured using a Sunrise ELISA reader (Tecan, Austria). All reported ELISA values were taken after 30 min substrate incubation and samples were considered positive when the absorbance at A_{405nm} values exceeded the mean of the healthy controls by at least factor of three (Wisler *et al.*, 2003).

Quantitative P. betae contents measured by nanobiosensor

Detection and relative quantification of *P. betae* content were done and confirmed in the same roots of baiting plants using specific nanobiosensor kit of *P. betae* (kindly provided by H. Safarpour, ABRII, Karaj, Iran). Nanobiosensor method was done based on Florescent Resonance Transfer Energy (FRET) using antibody attached quantom dots and GST conjugated rhodamine according to instructions of the kit producer (Safarpour *et al.*, 2012b). The test was conducted by first adding 250 µL of Tris-HCl buffer into each well. Then, 10 µL of the rhodamine-antigen solution was added. This was followed by an addition of 10 µL of the QDs-labeled antibodies solution. The baseline data were then recorded by a microplate reader (Tecan, Austria). The microplate reader was operated as follows: the excitation wavelength was set at 350 nm (the excitation wavelength of CdTe QDs) and the emission of the quencher's (rhodamine) was located at 580 nm. At the detection stage, the suspicious roots (0.1-0.5 mm thick) were mashed in Tris-HCl buffer (1 gr plant material/500 µL buffer). Twenty µL of the prepared extract was then added to the each well and the second round of data was

obtained. If no or negligible baseline shift (negative) was observed, the sample was marked as free of *P. betae* but significant baseline downward shift (positive) would reveal that the sample contained the pathogenic agents. The baseline shift measured for Immunodominant Membrane Protein (IMP) as the negative control (X±3SD), was used in differentiating healthy and infected samples.

Results

Identification of Viuliferous and Non-Viruliferous P. betae

Cystosori of *P. betae* were found in all soil samples by staining and microscopy studies (Fig 1). *P. betae* presence in the roots of plants grown in the soil samples tested was verified by PCR assays. Duplex PCR method was amplified two fragments of 454bp and 170bp in infected samples relating to rDNA region and specific region of the *P. betae,* respectively (Fig 2). Viruliferous *P. betae* isolate (Shiraz, Mashhad, Hamadan and Kermanshah,) and non-viruliferos *P. betae* isolates (Azarbayjan, Gorgan, Dezfool, Kerman, Karaj and Arak) were identified based on the presence of *P. betae* and BNYVV by DAS-ELISA in same lateral roots of sugarbeet plants. The results of this study indicated that microscopic detection of *P. betae* in agreement with serological and molecular methods (Table 1).

Table 1. Achieved results for identification of Viuliferous and Non-Viruliferous *P. betae* isolate using Light microscopy, DAS-ELISA and PCR methods.

Soil origine	Light microscopy (*P. betae*)	DAS-ELISA (*P. betae*)	PCR (*P. betae*)	DAS-ELISA (BNYVV)	Isolates Status
Shiraz	+	+	+	+	Viruliferous
Mashhad	+	+	+	+	Viruliferous
Azarbayjan	+	+	+	-	Non- viruliferous
Gorgan	+	+	+	-	Non- viruliferous
Dezfoul	+	+	+	-	Non- viruliferous
Kerman	+	+	+	-	Non- viruliferous
Karaj	+	+	+	-	Non- viruliferous
Hamadan	+	+	+	+	Viruliferous
Kermanshah	+	+	+	+	Viruliferous
Arak	+	+	+	-	Non- viruliferous
Control	-	-	-	-	-

Table 2. Analyzing viruliferous and non-viruliferous *P. betae* isolates with ELISA test and the constructed nanobiosensor.

Number	Soil samplesd	nanbiosensor (Baseline shiftc)[3]	P. betae ELISA (A405nm)	BNYVV ELISA (A405nm)
1	GST[1]	-662	0.99	-
2	Mashhad	-261	0.225	0.656
3	Shiraz	-236	0.171	0.457
4	Azarbaijan	-227	0.157	0.031
5	Kerman	-224	0.142	0.043
6	Dezfool	-219	0.123	0.055
7	Gorgan	-213	0.122	0.050
8	Kermanshah	-208	0.107	0.375
9	Arak	-200	0.127	0.051
10	Hamedan	-173	0.119	0.179
11	Karaj	-151	0.113	0.039
12	Healthy Sugar beet	-35	X+3SD=0.89	X+3SD=0.059
13	IMP[2](Negative control)	-18	-	-

1: Glutathion s-transferase recombinant protein, 2: Immunodominant Membrane Protein, 3: Change= Second run[b]- First run[a], a: First run at 580 nm [Buffer + (QDs-Ab) + (Rhodamine-GST)], b: Second Run at 580 nm [Buffer + (QDs-Ab) + (Rhodamine-GST) + sample], c: The baseline shift (-41±30 AU) associated with IMP has been used as a criterion to decide whether a sample is *P. betae*-infected, d: Each measurement is an average over five replicates.

Relative quantification of P. betae via Polymyxa specific GST ELISA and nanobiosensor

Polymyxa specific GST ELISA used for presence and relative quantity of *P. betae* displayed no significant differences in viruliferous and non-viruliferous *P. betae* progeny potential in different analyzed soils. However, Mashhad soil sample resulted in the highest *P. betae* concentration. Moreover, based on nanobiosensor results all Polymyxa positive samples were successfully detected and all of negative samples were found healthy by the nanobiosensor method as well (Table 2). Based on these results, the assay has sensitivity and a specificity of 100%. Moreover, in this study the results showed that the nanobiosensor data was in agreement with the results of the ELISA method (Table 2).

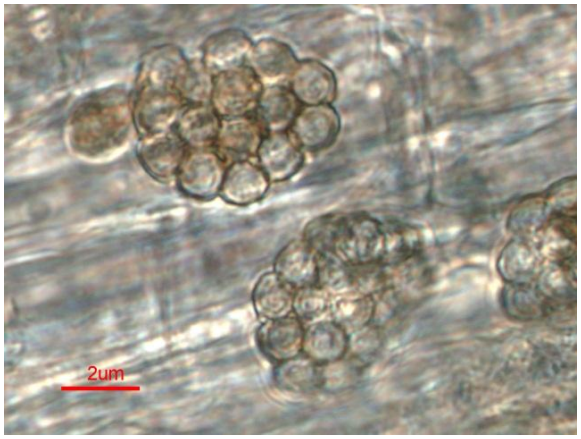

Fig. 1. Resting spores of *P. betae* in form of Cystosori

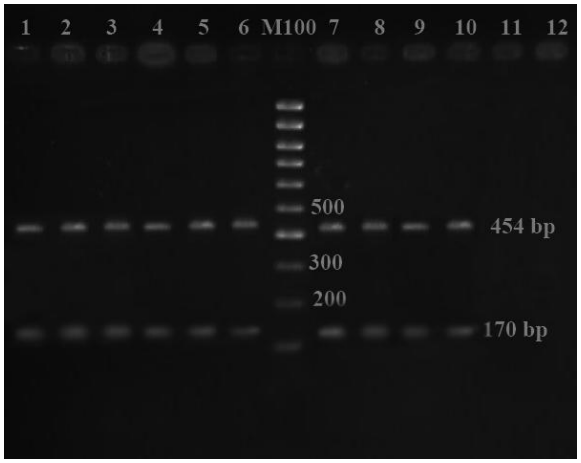

Fig. 2. Banding pattern of specific fragments of 454bp and 170bp relating to r DNA and specific regions of *P. betae* in infected roots. Lane (1-10): Simultaneous amplification of two specific band of *P. betae* in infected samples. Lane 12: Health control, lane 13: Negative control, M: standard molecular weight.

Discussion

Polymyxa betae is not truly considered as pathogens but as vector of sugarbeet viruses, and it plays crucial role in the epidemiology of viral disease (Pavli *et al*., 2011). Thus, little is known about the quantification of *P. betae* in the presence and absence of BNYVV. This study has shown aviruliferous *P. betae* is common in sugarbeet fields in Iran. This finding is in agreement with Yilmaz's (2010) observations which showed aviruliferous *P. betae* is common in sugarbeet fields in Turkey which could be expected to have a negative impact on sugarbeet yield, although many samples have no BNYVV. Indeed, the vector is distributed even more widely than the virus. For successful BNYVV-transmission a sufficient number of viruliferous *P. betae* zoospores in soil are a prerequisite (Asher *et al*., 2003). The vectors ability to multiply in sugarbeet roots plays a significant role regarding the possibility of resistance break. For some years, molecular methods have been applied to detect *P. betae* with the help of PCR. These methods have facilitated the identification of this root parasite, otherwise detected by microscopy or serological methods (Mutasa *et al*., 1993). The used methods in current paper have allow both precise detection and/or quantification of *P. betae* in roots of sugarbeet. These tools were developed on the basis of expressing of GST protein at high level in all the morphologically different stages of *P. betae* life cycle.

In this study obtained values of optical density of *P. betae* in soils with and without virus, from the quantitative DAS-ELISA, have no significantly difference. It seems, the *P. betae* content in lateral hair roots cannot be the only significant factor for the vector at successful transmission of BNYVV. In addition of differences *P. betae* isolates in the ability to take up and transmit BNYVV, other factors such as soil moisture and soil temperature also have impressive effect on rhizomania symptom severity.

Conclusion

Quantification could be used in epidemiological studies to determine the number of viruliferous *Polymyxa* cysts in soil after growing cultivars with different levels of viral resistance. Such data could also be useful in spreading modeling of this disease which would be applied for the decision-making of this kinds of diseases. Further research should be done to investigate the population of vector in different sugarbeet cultivar with viruliferous and aviruliferous *P. betae* isolates. In conclusion, regarding detection-limit for GST protein, its rapidness and also economic point of view nanobiosensor method for quantitative estimation of *P. betae* is suggested

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