

## Molecular Prediction of Pea Footrot Disease

<sup>1</sup>Ebimieowei Etebu and <sup>2</sup>A. Mark Osborn

<sup>1</sup>Department of Biological Sciences, Niger Delta University, Wilberforce Island,  
Bayelsa State, Nigeria

<sup>2</sup>Department of Biological Sciences, University of Hull, Cottingham Road,  
Hull, HU6 7RX United Kingdom

**Abstract:** PCR based assays were developed in this study to quantitatively predict pea footrot infections in agricultural soils prior to cultivation. Pea footrot disease due to *Nectria haematococca* (anamorph *Fusarium solani* f.sp. *pisi*) is linked to the presence of six pea pathogenicity (*PEP*) genes (*PDA1*, *PEP1*, *PEP2*, *PEP3*, *PEP4* and *PEP5*). Whilst molecular assays have been used recently to selectively detect these genes in soil-DNA, quantitative molecular assay has been extended to only the *PEP3* gene whose role in pea pathogenicity is yet unknown. In this research, PCR-based quantification assays were developed to quantify the two pea pathogenicity genes (*PDA* and *PEP5*) with identified roles in pea pathogenicity from soil-DNA obtained from fields with pea footrot histories. Results showed that the quantitative molecular assays developed herein were both efficient. Amplification efficiency of the Q-PCR assay for the *PDA* and *PEP5* gene were 97 and 89%, respectively. *PDA* and *PEP5* gene copy numbers were shown to vary significantly ( $p = 0.01$ ) between fields. However, the *PDA* gene copy numbers were relatively higher than those of the *PEP5* gene in agricultural fields. The genes, especially *PEP5* gene, were comparable to and positively correlated to the number of spores of pathogenic *N. haematococca*, and footrot disease. The *PDA* gene alone in soil could not cause footrot disease in peas after 8 weeks of planting; assays directed at it alone may therefore be insufficient to predict pea footrot disease. However, the molecular assay targeting the *PDA* alongside the *PEP5* gene offers the opportunity for quantitative prediction of pea footrot infections in agricultural soils prior to cultivation.

**Key words:** Footrot disease, *Nectria haematococca*, *PDA*, peas, pea pathogenicity genes, *PEP5*, soil-DNA

### INTRODUCTION

Footrot disease due to *Nectria haematococca* (anamorph *Fusarium solani* f.sp. *pisi*) is a globally, economically important disease of peas (McPhee, 2003). *N. haematococca* is pathogenic on all commercial processing pea cultivars (Hagedorn, 1991), and is responsible for yield losses up to 35-57% (Kraft, 1984; Oyarzun, 1993). There is currently no management practice capable of controlling the disease excepting the avoidance of fields with high disease potential (Oyarzun, 1993). Disease potential in many crops are generally positively related to initial inoculum density of plant pathogen(s) which in turn is known to be linearly correlated to disease incidence, onset, severity, and yield (Bhatti and Kraft, 1992; Navas-Cortés *et al.*, 2000; Rush and Kraft, 1986; Sugha *et al.*, 1994). Accurate assessment of the density of pathogenic forms of *N. haematococca* in soils, prior to pea cultivation, is therefore imperative to manage footrot disease.

The disease is linked to the presence of six pea pathogenicity (*PEP*) genes (*PDA1*, *PEP1*, *PEP2*, *PEP3*,

*PEP4* and *PEP5*) clustered on a conditional dispensable chromosome inherent in pathogenic forms of *Nectria haematococca* (anamorph *Fusarium solani* f. sp. *pisi*), and all highly virulent isolates have been shown to possess at least one homologue of each of the six genes except *PEP4* (Temporini and VanEtten, 2002). Until very recently, pathogenic forms in agricultural soils could not be ascertained without resorting to pathogenicity studies. Whilst molecular assays have been used recently to selectively detect these pea pathogenicity genes in soil-DNA (Etebu and Osborn, 2009), quantitative molecular assay has been extended only to the *PEP3* gene (Etebu and Osborn, 2010).

Although the *PEP3* gene is one of the genes linked with pea footrot disease, its role in pea pathogenicity is yet unknown (Idnurm and Howlett, 2001; Temporini and VanEtten, 2002), and unlike the rest of genes implicated with highly virulent strains of the fungus, it does not independently confer pathogenic properties to non-pathogenic isolates of *N. haematococca* that lack the conditional dispensable chromosome (Ciuffetti and VanEtten, 1996, Han *et al.*, 2001). Conversely, the *PDA*

and *PEP5* genes are able to independently confer pathogenic properties to non-pathogenic isolates of *N. haematococca* that lack the conditional dispensable chromosome (Ciuffetti and VanEtten, 1996, Han *et al.*, 2001). Additionally, of the pea pathogenicity genes, only the role of *PDA* gene in pea pathogenicity has been identified. Whilst *PDA* is responsible for detoxification of a phytoalexin (pisatin) via demethylation (VanEtten *et al.*, 1989; Delserone *et al.*, 1992), the *PEP5* gene is suggested to be involved in the efflux of pisatin (Han *et al.*, 2001). VanEtten *et al.* (2001) in their review, having observed that *N. haematococca* tolerates pisatin through degradative and non-degradative means, suggested that both types of tolerance mechanisms may operate in synergism during infection, and that elimination of both mechanisms may be required to make the fungus non-pathogenic. Molecular assays targeting the *PDA* and *PEP5* gene would therefore be desirable to selectively quantify pathogenic forms of *N. haematococca* in agricultural soils.

Hence in this research, molecular quantitative PCR-based assays have been developed to quantify the *PDA* and *PEP5* genes in soil-DNA extracted from agricultural fields with and without a prior footrot history. The quantitative molecular assay developed in this study is intended to quantitatively predict the likelihood of footrot infection of peas in agricultural fields prior to cultivation.

## MATERIALS AND METHODS

**Soil samples and total DNA extraction:** Soil samples previously obtained from five agricultural fields in the United Kingdom - field A, Lat. 52°51.1'N, long. 0°4.8'E; field B, Lat. 52°50.6'N, Long. 0°5.8'E; field C, Lat. 52°52.4'N, Long. 0°6.8'; field D, Lat. 52°50.6'N, Long. 0°11.4'E and field E, Lat. 52°52.6'N, Long. 0°0.8'W were used for experimentation. Also used were soil samples previously obtained from two parks-Park R, Lat. 53°23.0'N Long. 1°29.1'W and Park S, Lat. 53°22.6'N Long. 1°28.9'W. Whilst all the five agricultural fields had prior histories of footrot disease, the two parks neither had any history of footrot disease nor pea cultivation

Nucleic acids were extracted from 0.25 g of soil samples from all fields and parks using the PowerSoil DNA kit (MoBio, Carlsbad, CA) as according to the manufacturer's protocol. DNA was eluted into a final volume of 100 µL.

**Primer development and quantitative polymerase chain reaction (Q-PCR):** Two pairs of primers *PDAF3* (gct tcc atg atg gct tat ata cca) and *PDAR3* (cga tcg gta gga gtg aag aga ga), and *PEP5F3* (ggg ctt tac ggt tct ggc a) and *PEP5R2* (ggc ggt gta gac agg aag ag) were designed from sequences belonging to *N. haematococca*

retrieved from GenBank database (accession numbers L20976) using ABI Primer express software (Applied Biosystems, California, USA) to target the *PDA* and *PEP5* genes, respectively.

Using these pairs of primers (*PDAF3/PDAR3* and *PEP5F3/PEP5R2*), the *PDA* and *PEP5* genes present in soil samples of the different agricultural fields, commercial topsoil, and Parks were separately quantified. Firstly, a PCR was run to amplify partial sequences of the *PDA* and *PEP5* genes of isolate C31 recently described by Etebu and Osborn (2009).

With these primer sets the amplicon sizes for *PDA* and *PEP5* partial gene sequences were 70 and 162 base pairs, respectively. The PCR products were visualized by 1-2% agarose gel electrophoresis, and the sizes were verified by comparing with Hyperladder V (Bioline, London, UK). The concentrations of the PCR products (referred herein as neat standards) were thereafter determined through spectrophotometric absorbance measurement at 260 nm wavelength, using Eppendorf BioPhotometer (Helena BioSciences, Sunderland, UK). Gene copy numbers of the neat standards were calculated as according to Smith *et al.* (2006) from the formula:

$$\text{Gene numbers} = [6.023 \times 10^{23} (\text{copies/mol-1}) \times \text{concentration of standard (g/}\mu\text{L)}] / [\text{MW (g/mol-1)}]$$

where;  $6.023 \times 10^{23}$  = avogadro's number; and the average Molecular Weight (MW) of one base pair of double stranded DNA (dsDNA) equals 660Da.

Having determined the gene copy numbers resulting from PCR, a range of standards with varying gene copy numbers were obtained through serial dilutions of the neat standard.

The various dilutions of the neat standard, DNA samples extracted from agricultural soils with unknown gene copy numbers and No Template Control (NTC) were amplified together in separate wells in a 96-well plate using the same set of primers employed in the determination of gene numbers obtained from PCR amplification of isolate C31 (neat standard). Amplification of all samples were done in triplicates, each reaction well containing 5 µL of template DNA, 0.5 µL sensimix, 0.5 µL SYBR green (Applied Biosystems, California, USA), 200 nM of each primer and made up to 25 µL with sterile distilled water. PCR and fluorescence detection was performed using an Applied Biosystem ABI 7700 Sequence Detection System (Applied Biosystems, California, USA).

Cycling conditions aimed at quantifying *PDA* gene copy numbers were first held at 50°C for 2 min, and then the amplification process was programmed to begin by an initial denaturation step for 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec, 50°C for 15 sec and 60°C for 45 sec.

Those aimed at quantifying the *PEP5* gene were similar to the latter with an initial denaturation step for 10 min at 95°C; followed by 40 cycles of 95°C for 15 sec and 60°C for 75 sec.

At the end of the thermal cycling process, the threshold cycle (Ct), was determined for the unknown sample-DNA, as well as for the range of standards constructed from known amounts of the genes of interest (Smith *et al.*, 2006). Following the determination of the threshold cycle, simple linear regression graphs (standard curves) were plotted between the Ct values and their corresponding known amounts of the pea pathogenicity genes contained in the standards. The slopes, y-intercepts and the coefficients of determination ( $r^2$ ) were determined as according to Smith *et al.*, (2006). Also the efficiency of the amplification reaction ( $E$ ) was calculated from the equation  $E = 10^{[-1/\text{slope}]-1}$  (Arezi *et al.*, 2003; Smith *et al.*, 2006), and expressed as a percentage.

Gene copy numbers contained in the agricultural soil-DNA samples were thereafter extrapolated from the standard curve through their Ct values. Gene copy numbers from all fields were thereafter log transformed and subjected to ANOVA (Statistical package for social science, version 12.0).

**Validation of pea pathogenicity (*PDA* and *PEP5*) gene numbers as estimates of the population of pathogenic forms of *N. haematococca* in soils:** The suitability of the quantitative molecular assays developed in this study to measure the inoculum density of pea pathogenic *N. haematococca* in agricultural soils was tested as according to Etebu and Osborn (2010). Commercial top soil was separately infested in 4 replicates with tap water and varying amounts of conidial suspension of a tested pathogenic strain, C31 described by Etebu and Osborn (2009; 2010) and labelled D0, D2, D3, D4 and D5 respectively. Whilst all 4 replicates of top soil labelled D0 had no fungal spore, those labelled D2, D3, D4 and D5 had 1.04E+02, 1.04E+03, 1.04E+04 and 1.04E+05 fungal conidia g<sup>-1</sup>soil, respectively. Aliquots of soil samples were collected from all replicates of all treatments (D0-D5), and the *PDA* and *PEP5* genes were separately quantified as described earlier in this study. Copy numbers of the two genes were compared to the number of spores (conidia) introduced into the commercial topsoil.

**DNA sequencing:** To further investigate the *PDA* gene present in commercial topsoil prior to artificial infestation with fungal suspension (treatment D0), an endpoint PCR was performed using PDAF2 (TGC GTC TCT CTT CAC TCC TAC CG) and PDAR2 (CGA AGA GTG TGC AGA GTA CGT GG) primers pairs according to Etebu and Osborn (2009). Clone libraries of partial *PDA* gene products amplified from soil DNA were constructed in pCR4-TOPO (Invitrogen, Paisley, U.K.) Inserts from white transformants were then amplified using vector primers (T3 and T7). Amplicons from clones were

purified as above prior to sequencing of both strands using the BigDye terminator V1.1 cycle sequencing kit (Applied Biosystems, Warrington, U.K.) and electrophoresis on an ABI 3730 automated DNA capillary sequencer (Applied Biosystems). Sequences were analysed using Sequencing Analysis software version 5.1 (Applied Biosystems), and manually edited using Chromas 2.1 (Technelysium.com). Edited sequences were then compared to sequences in GenBank using BLAST (1). Phylogenetic analysis of gene sequences was performed using Bioedit (Hall, 1999), Genedoc (Nicholas and Nicholas, 1997) and Phylip (Felsenstein, 1989) software.

## RESULTS

**Molecular quantification of pea pathogenicity gene (*PDA* and *PEP5*) in agricultural soils:** The abundance of two pea pathogenicity genes (*PDA* and *PEP5*) in agricultural fields with a prior footrot history was estimated using quantitative PCR (Q-PCR) techniques. The *PDA* and *PEP5* gene numbers in the different agricultural fields was quantified using the standard curve method (Fig. 1 and 2, respectively).

The amplification efficiency ( $E$ ) which was calculated from the equation  $E=10^{[-1/\text{slope}]-1}$ , and expressed as a percentage was found to be 97% and 89% for the *PDA* and *PEP5* genes, respectively (Fig. 1 and 2). Molecular analyses showed significant ( $p = 0.01$ ) differences between agricultural fields with respect to all two (*PDA* and *PEP5*) pea pathogenicity gene numbers (Table 1). None of the two genes were detected from the two parks which had neither history of footrot disease nor pea cultivation. Log *PDA* gene numbers across the five agricultural field soils ranged from 3.35 to 4.10 g<sup>-1</sup>Soil

Table 1: Molecular estimation of pea pathogenicity gene numbers in soil-DNA extracted from agricultural fields with a prior footrot history using the SYBR green method of quantitative PCR

Field	Pea crop interval <sup>1</sup> (years)	Mean log pea pathogenicity gene numbers (g <sup>-1</sup> Soil) (n = 3)	
		<i>PDA</i>	<i>PEP5</i>
A	12	3.65±0.05 <sup>abc</sup>	2.54±0.01 <sup>ab</sup>
B	5	4.10±0.16 <sup>c</sup>	3.27±0.13 <sup>bc</sup>
C	Not given	4.06±0.12 <sup>bc</sup>	3.32±0.18 <sup>c</sup>
D	>8	3.35±0.05 <sup>a</sup>	2.37±0.09 <sup>a</sup>
E	8	3.60±0.08 <sup>ab</sup>	2.39±0.27 <sup>a</sup>
R	na	Not detected	Not detected
S	na	Not detected	Not detected
NTC	na	Not detected	Not detected
	Mean	3.75	2.78

<sup>1</sup> Pea crop interval refers to the last year of pea cultivation for each field. Fields with same letters were not significantly ( $p = 0.05$ ) different for every corresponding gene. Log gene numbers were calculated from the following standard curves: for *PDA*,  $r^2 = 0.99$ , amplification efficiency = 97%, intercept = 40.421, Ct of NTC = 38.54; for *PEP5*,  $r^2 = 0.99$ , amplification efficiency = 89%, intercept = 37.134, Ct of NTC = 40

Table 2: The relationship between numbers of conidia of *Nectria haematococca* (anamorph *Fusarium solani* f. sp. *pisi*) and pathogenicity gene copy numbers estimated by quantitative PCR and their effect on footrot disease and total plant dry weight of peas after 8 weeks of planting

Treatment	Initial log number of conidia (g <sup>-1</sup> soil) added into soil	Initial log mean pea <sup>1</sup> pathogenicity gene numbers (g <sup>-1</sup> soil)			Mean <sup>2</sup> footrot disease	Plant dry <sup>2</sup> weight (g)
		<i>PDA</i> -T	<i>PDA</i> -S	<i>PEP5</i>		
D0	Control	3.51 <sup>a</sup>	-	-	0.50 <sup>a</sup>	0.74 <sup>b</sup>
D2	2.01	4.34 <sup>b</sup>	2.62 <sup>a</sup>	2.53 <sup>a</sup>	2.25 <sup>b</sup>	0.44 <sup>ab</sup>
D3	3.01	5.22 <sup>c</sup>	3.50 <sup>b</sup>	3.70 <sup>b</sup>	2.50 <sup>b</sup>	0.49 <sup>ab</sup>
D4	4.01	6.30 <sup>d</sup>	4.58 <sup>c</sup>	4.80 <sup>c</sup>	3.25 <sup>b</sup>	0.39 <sup>a</sup>
D5	5.01	7.23 <sup>e</sup>	5.53 <sup>d</sup>	5.56 <sup>d</sup>	4.00 <sup>c</sup>	0.34 <sup>a</sup>

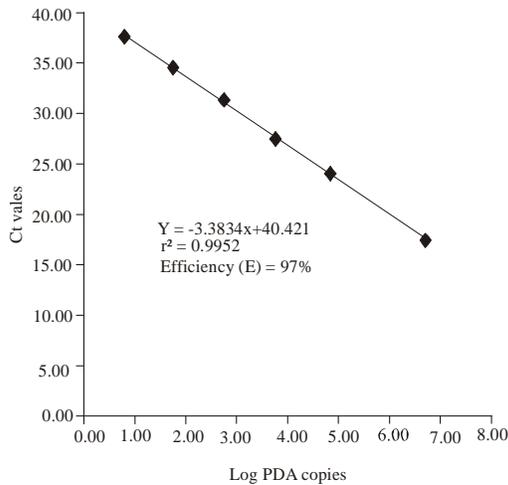


Fig. 1: Linear regression of the Ct values derived by quantitative PCR amplification of known amounts of *PDA* gene numbers versus log of the initial *PDA* gene copy numbers. *PDA* gene was generated from isolate C31 using *PDA*F3 and *PDA*R3 primers

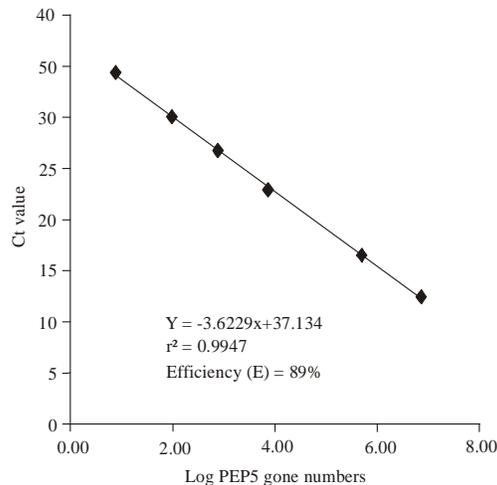


Fig. 2: Linear regression of the Ct values derived by quantitative PCR amplification of known amounts of *PEP5* gene numbers versus log of the initial *PEP5* gene numbers. *PEP5* gene was generated from isolate C31 using *PEP5*F3 and *PEP5*R2 primers

(mean 3.75, n = 3; Table 1). Statistical analysis showed that the *PDA* gene was most abundant in field B (4.10±0.16, n = 3). It was observed to have more ( $P=0.05$ ) *PDA* genes than field D and E (Table 1). Although field B was found to have the highest number of *PDA* genes, their abundance was not significantly ( $p = 0.05$ ) different from fields A (3.65±0.05) and C(4.06±0.12). Field D had the lowest number of *PDA* genes (3.35±0.05). The difference in *PDA* gene numbers between Field D and those of A and E were not significant ( $p = 0.05$ ).

Mean log *PEP5* gene numbers ranged from 2.37 to 3.32 g<sup>-1</sup>Soil (mean 2.78; n = 3). The abundance of this gene was also observed to vary significantly ( $p = 0.01$ ) amongst the different agricultural fields sampled (Table 1). The *PEP5* gene was highest in field C (Table 1). It had more *PEP5* gene numbers ( $p = 0.05$ ) than fields A, D and E. The latter fields had the lowest *PEP5* gene numbers, and were not significantly different from one another (Table 1). Although, the *PEP5* gene numbers were highest in field C, the difference between *PEP5* gene numbers in fields C and B was not significant ( $p = 0.05$ ) just like the *PDA* gene. The latter field was observed to have significantly ( $p = 0.05$ ) more *PEP5* genes than D and E. Also as was observed with the *PDA* gene, the *PEP5* gene was not detected in soil-DNA obtained from parks R and S which neither had any history of footrot disease nor pea cultivation.

**Relationship between pathogenicity gene numbers and number of conidia of *N. haematococca* in soil:** A linear regression graph showing the numbers of two pathogenicity genes (*PDA*, and *PEP5*) estimated through Q-PCR, plotted against the number of conidia inoculated into soil showed a very strong ( $r^2 > 99\%$ ) positive relationship (Table 2, Fig. 3). Gene copy numbers for all the genes investigated were directly proportional to the number of spores per gram of commercial topsoil. Although molecular quantification results of gene copy numbers for both pathogenicity genes (*PDA* and *PEP5*) increased significantly ( $p = 0.05$ ) in direct proportion to the number of spores of *F. solani* inoculated into commercial topsoil (Table 2), only the mean log gene copy numbers of the *PEP5* were comparable with the log

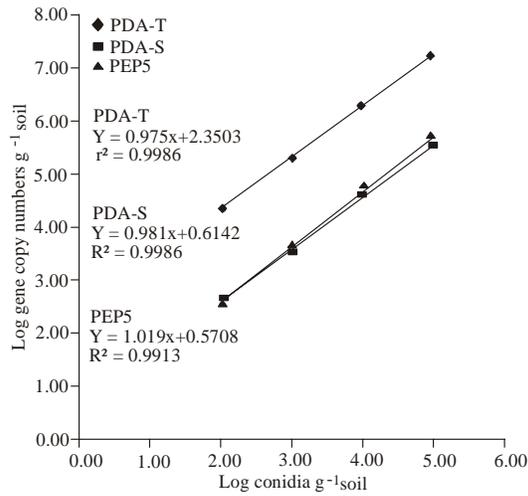


Fig. 3: Linear regression of conidial numbers of *N. haematococca* in topsoil versus pea footrot pathogenicity gene numbers estimated by Q-PCR. Log gene numbers were calculated from the following standard curves: for *PDA*,  $r^2 = 0.99$ , Amplification efficiency = 99%, intercept = 37.134, Ct of NTC = 40; *PDA-T* = Total number of *PDA* genes; *PDA-S* = Number of *PDA* genes inoculated into soil = Total number of *PDA* genes in inoculated soil - numbers of *PDA* genes in uninoculated soil

of conidia density in soil, the *PDA* gene copy numbers were clearly higher. For example treatment D5 which had topsoil with an inoculum density of  $1.04E+05$  spores ( $g^{-1}$  Soil), and whose log (spore concentration) translates to 5.01 had 7.23 and 5.56 as mean log gene numbers for the *PDA* and *PEP5* genes, respectively. An initial PCR investigation of the different soil treatments showed positive amplification of the topsoil treatments (including the control, treatment D0) (Data not shown). However, subtracting the log gene copy numbers of the control treatment D0 from treatments D2, D3, D4 and D5 gave the log inoculum density as 2.62, 3.50, 4.58 and 5.53, respectively, comparable to the corresponding log of initial inoculum density of the *PEP5* gene copy numbers (Table 2).

Neighbour joining phylogenetic analysis of 33 *PDA* gene partial sequences obtained from the commercial topsoil and two *PDA* sequences (L20976 and X73145) from *F. solani* f. sp. *pisi* (Miao *et al.*, 1991a, b), available in the GenBank database showed that all 33 sequences were most (>99%) closely related to either of the two *PDA* sequences retrieved from the GenBank database (Fig. 4).

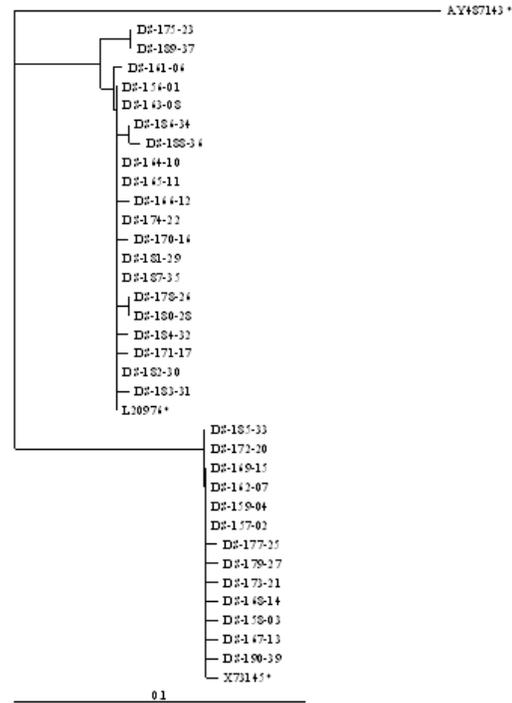


Fig. 4: Neighbor-joining tree produced from ClustalW alignment of partial *PDA* gene sequences of amplified from soil-DNA from topsoil generated from *PDAF2* and *PDAR2* primers and compared to those obtained in GenBank\*: *PDA* sequences derived from GenBank, L20976 and X73145 derived from *F. solani* f. sp. *pisi*; AY487143 derived from *F. oxysporum* f. sp. *pisi*

**The effect of inoculum density of pathogenic *N. haematococca* in soil on pea footrot disease:** Footrot disease was dependent on initial inoculum or spore density available in soil to cause infection. Generally the greater the number of spores of pathogenic *Fusarium solani* in soil the more severe the footrot disease in peas (Table 2, Fig. 5). This trend was also observed with respect to both *PDA* and *PEP5* pea pathogenicity genes estimated from soil-DNA (Table 2). All four topsoil treatments (ie, statistical treatments) D2, D3, D4 and D5, whose log (spore concentration) were 2.01, 3.01, 4.01 and 5.01, respectively (Table 2) showed that molecular estimation of the two genes also revealed a similar trend of significant ( $p = 0.05$ ) increase in number. These in turn, led to a significantly ( $p = 0.05$ ) higher severity of footrot disease in peas than treatment D0 (Table 2, Fig. 5). Treatment D5 caused footrot in peas up to 4.00 in a 0-5 disease scale after 8 weeks of planting (Table 2). Footrot disease at this density was significantly ( $p = 0.05$ ) higher than the control topsoil (treatment D0) that was treated with water only without conidia and treatment (D2). This control treatment caused footrot disease index values of



Fig. 5: Effect of initial inoculum density of *N. haematococca* on footrot disease severity in Greenshaft peas after 8 weeks of planting. (Left to right Control (tap water),  $1.04E+02$ ,  $1.04E+03$ ,  $1.04E+04$  and  $1.04E+05$  conidia ( $g^{-1}$  soil))

0.5 during the period of 8 weeks of planting. Although treatment D5 clearly caused the most severe pea footrot disease, it was nonetheless not significantly ( $p = 0.05$ ) different from disease caused by treatments D3 and D4 after 8 weeks of planting. Similarly, differences in footrot disease between treatments D2, D3 and D4 were not significant ( $p = 0.05$ ) (Table 2).

Statistical ( $p = 0.05$ ) differences were observed within treatments which had varying concentrations of spores added to topsoil with respect to total pea dry weight matter. Peas planted in treatment D0 had the greatest pea dry weight matter. Its mean dry weight matter was 0.74 g, and was significantly ( $p = 0.05$ ) greater than those planted in treatments D4 and D5. Differences in pea dry weight between peas planted in treatment D0, D2 and D3 were not significant ( $p = 0.05$ ). Mean pea dry weight due to treatments D2 and D3 were 0.44 and 0.49 g, respectively, and were not significantly ( $p = 0.05$ ) different from those of treatments D4 and D5 which had dry weight of peas of 0.39 and 0.34 g, respectively after 8 weeks (Table 2).

Pea footrot disease was observed to be related to pea dry weight matter (Table 2). Footrot disease was generally negatively related to total pea dry weight matter after 8 weeks of planting in soil having varying amounts of inoculum load of pathogen. Pearson correlation of footrot disease and total plant dry weight across all 20 data points showed that the two parameters were significantly ( $p = 0.01$ ) negatively correlated ( $r^2 = -0.73$ ) (data not shown).

## DISCUSSION

**Molecular quantification of pea pathogenicity genes (*PDA* and *PEP5*) in agricultural soils:** The *PDA*, and *PEP5* gene copy numbers in the different agricultural fields investigated were quantified using the standard

curve method (Fig. 1 and 2 respectively) because amongst the several procedures and proposals available in scientific literature on ways to analyse and validate data generated from quantitative PCR, the standard curve method is considered to be the simplest, and under the right conditions, the most precise method available (Marino *et al.*, 2003).

The number of the *PDA* and *PEP5* genes were observed to be statistically different ( $p = 0.05$ ) between fields. This shows that the molecular assay developed in this work could be used to discriminate between agricultural fields with respect to abundance of pea pathogenicity genes, and by extension, the number of pathogenic strains (inoculum load) available in soil to cause footrot infection on peas. A recent work by Etebu and Osborn (2010), using same soil samples also showed comparable differences in the *PEP3* gene copy numbers between the different agricultural fields. Furthermore, it is interesting to note that the *PEP3* gene copy numbers obtained from that study were comparable to the *PEP5* gene copy numbers obtained from this study. This shows that the *PEP5* gene could be a target gene to determine the number of pathogenic forms of *Nectria haematococca* in agricultural soils.

Gene copy numbers for the *PDA* gene were higher than those of the *PEP5* gene. Although the relative abundance of pea pathogenicity genes in agricultural fields prior to cultivation have not been reported until very recently with the *PEP3* gene (Etebu and Osborn, 2010), the *PDA* gene was expected to be more abundant in agricultural soils with a prior footrot history. In an earlier experiment, Etebu and Osborn (2009) showed that whilst, 33.3% of isolates obtained from the agricultural fields reported herein had the *PDA* gene, only 20% of the isolates had the *PEP5* gene. All isolates which had the *PEP5* gene also possessed the *PEP3* gene, hence it is not surprising to see that the copy numbers of the *PEP5* gene obtained from this study was also comparable to the *PEP3* gene copy numbers obtained in an earlier work (Etebu and Osborn, 2010) since both studies used the same soil samples.

### **Relationship between pea footrot pathogenicity gene numbers and number of conidia of *N. haematococca* in soil:**

Having demonstrated the capability of the molecular assay to differentially amplify pea footrot pathogenicity genes from soil-DNA of different fields without recourse to cultural techniques, a separate experiment was carried out to validate these results. Results from this experiment clearly showed that the observed differences in gene numbers between the different fields, arising from the molecular assays, were indeed due to significant differences in the number of pathogenic strains of *Fusarium solani* f. sp. *pisi* available in these soils to cause pea footrot disease. A simple linear regression graph

plotted between the pea pathogenicity genes (*PDA* and *PEP5*) in soil-DNA estimated through Q-PCR versus the number of conidia added to soil prior to DNA extraction showed very strong ( $r^2 > 0.99$ ) positive relationship (Fig. 2). The gene copy numbers for all two genes were directly proportional to the number of spores contained in the suspension used in infesting the commercial topsoil.

Numerous workers have shown that molecular-based assays could indeed be a reliable measure of the number of whole organisms (Johnsen *et al.*, 1999; Lechner and Conrad, 1997; Cordier *et al.*, 2007; Kabir *et al.*, 2003; Mahuku and Platt, 2002; Mahuku *et al.* 1999). These findings notwithstanding, plant pathogenicity genes with identified roles in pathogenic fungi have not been quantified by molecular methods in environmental samples and shown to correlate with actual inoculum numbers until now. The role of *PEP3* gene recently targeted in molecular quantification experiments, and shown to correlate with actual number of pathogenic forms of *Fusarium solani* f. sp. *pisi* in soil (Etebu and Osborn, 2010) is yet unknown.

Although the gene numbers for the two pea pathogenicity genes (*PDA* and *PEP5*) increased significantly ( $p = 0.05$ ) in direct proportion to the number of spores of *F. solani* inoculated into commercial topsoil (Table 2 and Fig. 3), only the mean log gene copy numbers of the *PEP5* gene were comparable to the log number of conidia for all given concentrations of spores in soil. Those recorded for the *PDA* gene were clearly higher. For example treatment D5 which had topsoil with a log conidial density of 5.01 ( $\text{g}^{-1}$  Soil), had 7.23, and 5.56 as mean log gene copy numbers  $\text{g}^{-1}$  Soil for the *PDA* (*PDA*-T), and *PEP5* genes, respectively (Table 2). Apparently these results seemed to suggest that the *PDA* gene was either overestimated or the commercial topsoil had the *PDA* gene prior to experimentation. To this end, an endpoint PCR targeting the *PDA* gene was carried using the primers *PDAF2* and *PDAR2* as described by Etebu and Osborn (2009). Results showed that there was a positive amplification of all treatments of topsoil infested with various amounts of conidia of *N. haematococca* (including the control, treatment D0) (Data not shown). This confirmed the presence of the *PDA* genes in the commercial topsoil prior to experimentation, or at least the molecular assay generated a product of the same amplicon size expected of the *PDA* gene. This explains why the *PDA* gene estimates generated from the molecular assay were higher than the number of conidia introduced into the soil. The log *PDA* (*PDA*-S) gene copy numbers became comparable to the log *PEP5* gene copy numbers when the log number of the *PDA* genes estimated in the control soil was subtracted from the total *PDA* gene numbers (*PDA*-T) of the other treatments (Treatments D2, D3 D4 and D5) (Fig. 3 and Table 2).

To further investigate the presence of the *PDA* genes in the commercial topsoil prior to experimentation, the amplified products of treatment D0 were cloned and sequenced. BLAST and neighbour joining phylogenetic analyses showed that 20 out of 33 (60.60%) of the sequences were most ( $\geq 99\%$ ) closely related to the *PDA*<sup>H</sup> (accession number L20976) form of the *PDA* gene, and 13 of the 33 clones (39.40%) had sequences most closely related to the *PDA*<sup>L</sup> (accession number X73145) form of the *PDA* gene (Fig. 4). Pathogenicity of *N. haematococca* has been partly attributed to the presence of the *PDA* gene. This gene encodes a protein that is able to demethylate the phytoalexin, pisatin, produced by peas on infection. Natural isolates of *N. haematococca* vary quantitatively in pisatin demethylating ability, and as a result, three whole cell phenotypic groups have been classified (Matthews and VanEtten 1983), which include *PDA*<sup>-</sup>, *PDA*<sup>L</sup> and *PDA*<sup>H</sup>. The first group lack the ability to detoxify pisatin (*PDA*<sup>-</sup>). The second group produce low levels of pisatin after long exposure to pisatin (*PDA*<sup>L</sup>) while the third group rapidly produces moderate to high levels of *PDA* on exposure to Pisatin (*PDA*<sup>H</sup>) (Mackintosh *et al.*, 1989). Isolates that possess the *PDA*<sup>H</sup> form the *PDA* gene were thought to be more pathogenic than those that possess the *PDA*<sup>L</sup> form (Matthews and VanEtten 1983; Mackintosh *et al.*, 1989). However, a more recent work by Etebu and Osborn (2009) have shown that natural isolates that possess the *PDA*<sup>L</sup> form of the *PDA* gene could also be highly pathogenic, if such isolates possess other genes such as *PEP3* and *PEP5* in addition. The phylogenetic results obtained from this work (Fig. 4) showed that the commercial topsoil used in this work had *PDA* genes native to *Fusarium solani* f. sp. *pisi*, prior to experimentation but could not cause an appreciable disease in peas. The mean pea footrot disease index value after 8 weeks of planting was 0.50. Soil samples with footrot disease index value of up to 0.86 after 5 weeks of planting are adjudged as having a very low risk of footrot disease (Biddle, 1984). The apparent lack of footrot disease in peas planted on the control commercial topsoil, notwithstanding the presence of the *PDA* gene prior to planting, showed that pea pathogenic forms of *Fusarium solani* f. sp. *pisi*, requires the *PEP3* and/or *PEP5* gene in addition to the *PDA* gene, to achieve maximum levels of virulence in peas (Etebu and Osborn, 2009; Han *et al.*, 2001; Temporini and VanEtten, 2002; Wasmann and VanEtten, 1996). Having said this, the aim of this research work was to show that pea pathogenicity gene copy numbers generated through the quantitative molecular assays developed in this work correlated positively to the inoculum density of virulent *N. haematococca*. This was clearly achieved, and thus the assays offer a more reliable method for the quantification of inoculum density of pea pathogenic *N. haematococca* in soils than culture dependent assays or molecular assays

directed at gene sequences other than those linked with pea footrot disease.

The accurate quantification of the inoculum density of a pathogen in soil is essential for disease forecast and for assessing the outcome of control measures (Cullen *et al.*, 2001, 2002; Goud and Termorshuizen, 2003). This underscores the prospective expediency of the molecular-based assays developed in this study, as they selectively quantified pathogenic strains of *N. haematococca* in soil by targeting pea pathogenicity (*PDA* and *PEP5*) genes.

#### **The effect of inoculum density of pathogenic *N. haematococca* in soil on pea footrot disease and yield:**

Generally, high number of spores of pathogenic *Fusarium solani* in soil, showed a correspondingly more severe footrot disease in peas (Table 2; Fig. 5). Inoculum density was linearly correlated to pea footrot disease index. All four portions of topsoil (treatments D2, D3, D4 and D5) separately inoculated with  $1.04 \times 10^2$ ,  $1.04 \times 10^3$ ,  $1.04 \times 10^4$  and  $1.04 \times 10^5$  conidia ( $\text{g}^{-1}$  soil) respectively had a significantly ( $p = 0.05$ ) higher number of pathogenicity genes which, in turn was responsible for a correspondingly more severe footrot disease in peas than in treatment D0 (Table 2, Fig. 3 and 5). Several works have shown that initial inoculum density of plant pathogen(s) is linearly correlated to disease incidence, onset, and severity and in some cases ultimately to yield of various crops (Bhatti and Kraft, 1992; Navas-Cortés *et al.*, 2000; Sugha *et al.*, 1994). In particular, Rush and Kraft (1986) showed that root rot due to *N. haematococca* (anamorph *F. solani* f. sp. *pisi*) was related to inoculum density, especially when peas are sown  $\leq 10$  cm deep in soil or when soil was thoroughly mixed with inoculum of *F. solani* f. sp. *pisi*. Root length, plant height and leaf areas were also observed to be significantly lower than in control pea plants growing in soil, not infested with *F. solani* f. sp. *pisi*.

The effect of inoculum density of plant pathogens on disease has been demonstrated in other plants. *Fusarium oxysporum* f. sp. *ciceris*, known to cause Fusarium wilt in chickpea have been variously demonstrated to show that an increase in inoculum density caused an exponential reduction in disease onset, and increased incidence and severity (Bhatti and Kraft, 1992; Navas-Cortés *et al.*, 2000; Sugha *et al.*, 1994). Bhatti and Kraft (1992) reported that an inoculum density of  $5.00\text{E}+03$  propagules ( $\text{g}^{-1}$  soil) of *F. oxysporum* f. sp. *ciceris* caused the most severe wilt of chickpea.

The most severe disease recorded in this work was with topsoil inoculated with  $1.04\text{E}+05$  conidia ( $\text{g}^{-1}$  soil). A total pea crop failure was observed at this initial inoculum density (Fig. 5). Mean Pea footrot disease index recorded after 8 weeks of planting was 4.00. Biddle (1984) reported that agricultural fields with footrot disease indices of values between 1.26 and 2.00 in peas

planted after 5 weeks, or when plants begin to produce flowers, indicated a medium risk of footrot. Similarly, values between 2.1 and 2.75 indicated a high risk while values above 2.75 would be uneconomic to grow peas. It therefore follows that log gene copy numbers of the *PEP5* gene of up to 2.00-3.00 would indicate a medium to high risk in these soils. Agricultural fields with estimated log numbers of pathogenicity genes, especially, *PEP5*, of  $\geq 4.00$  may indicate a high risk to footrot. Similar assertions have been made with respect to the *PEP3* gene (Etebu and Osborn, 2010). An inoculum density of  $1.04\text{E}+02$  conidia ( $\text{g}^{-1}$  soil) was the lowest concentration used in this experiment, and it caused a significantly greater footrot disease in peas after 8 weeks of planting than those planted in topsoil, not infested with pathogen (Table 2; Fig. 5).

The relationship between footrot disease and total plant dry weight was investigated, as the latter has been demonstrated to be a suitable measure of pea yield (Hobbs and Mahon, 1982). Total plant dry weight was negatively related to footrot disease index (Table 2). Oyarzun (1993) observed a proportional decrease in yield of peas as root rot in fields increased. Experiments have shown that a 1 point difference in footrot disease index value translates to a yield difference of approximately 1 ton/ha, even without a corresponding obvious change in above-ground disease symptoms (Biddle, 1984; Oyarzun, 1993). The results presented in this work, are in agreement with the findings of previous workers and unequivocally show that inoculum density contributes to footrot disease. Accurate footrot disease forecast therefore requires accurate quantification of the inoculum density of *N. haematococca* in soil. Whilst different workers have variously shown the need for the development of molecular quantification assays as a prerequisite for determining the soil inoculum threshold levels necessary for disease development in a host-pathogen relationship (Cullen *et al.*, 2001, 2002; Goud and Termorshuizen, 2003), this has not been achieved with footrot disease in peas until very recently with the *PEP3* gene (Etebu and Osborn, 2010) whose role in pea pathogenicity is yet unknown (Idnurm and Howlett, 2001; Temporini and VanEtten, 2002).

## **CONCLUSION**

In this research, PCR-based quantification assays were developed to quantify the two pea pathogenicity genes (*PDA* and *PEP5*) whose roles in pea pathogenicity have been identified. Although the *PDA* gene has been identified as an essential pathogenic determinant required for pea footrot disease numerous works and results from this work showed that the *PDA* gene alone could not cause appreciable footrot disease in peas even after 8 weeks of planting. Assays directed at the *PDA* gene could

therefore serve only as a preliminary diagnostic measure. Molecular assay developed in this work, targeting the *PEP5* gene may be required to predict pea footrot infections in agricultural soils prior to cultivation.

#### ACKNOWLEDGMENT

The authors would like to thank the Bayelsa State Government of Nigeria for funding the research, and Anthony Biddle (U.K. Processors and Growers Research Organisation) and Richard Fitzpatrick (Holbeach Marsh Cooperative) for their assistance in facilitating access to agricultural fields in East Anglia.

#### REFERENCES

- Arezi, B., W. Xing, J.A. Sorge and H.H. Hogrefe, 2003. Amplification efficiency of thermostable DNA polymerases. *Anal. Biochem.* 321: 226-235.
- Bhatti, M.A. and J.M. Kraft, 1992. Effects of inoculum density and temperature on foot rot and wilt of chickpea. *Plant Dis.*, 76: 50-54.
- Biddle, A.J., 1984. A prediction test for pea footrot and the effect of the previous legumes. *British Crop Protection Conference-Pests and Diseases*, pp:773-777.
- Ciuffetti, L.M. and H.D. Van-Etten, 1996. Virulence of a pisatin demethylase-deficient *Nectria haematococca* MPVI isolate is increased by transformation with a pisatin demethylase gene. *Mol. Plant-Microbe Interact.* 9: 787- 792.
- Cordier, C., V. Edel-Hermann, F. Martin-Laurent, B. Blal, C. Steinberg and C. Alabouvette, 2007. SCAR-based real time PCR to identify a biocontrol strain (T1) of *Trichoderma atroviride* and study its population dynamics in soils. *J. Microbiol. Meth.*, 68: 60-68.
- Cullen, D.W., A.K. Lees, I.K. Toth and J.M. Duncan, 2001. Conventional PCR and real-time quantitative PCR detection of *Helminthosporium solani* in soil and potato tubers. *Euro. J. Plant Pathol.*, 107: 387-398.
- Cullen, D.W., A.K. Lees, I.K. Toth and J.M. Duncan, 2002. Detection of *collectotrichum coccodes* from soil and potato tubers by conventional and quantitative real-time PCR. *Plant Pathol.*, 51: 281-292.
- Delserone, L.M., D.E. Matthews and H.D. Van-Etten, 1992. Differential toxicity of enantiomers of maackiain and pisatin to phytopathogenic fungi. *Phytochem.*, 31: 2933-2935.
- Etebu, E. and A.M. Osborn, 2009. Molecular assays reveal the presence and diversity of genes encoding pea footrot pathogenicity determinants in *Nectria haematococca* and in agricultural soils. *J. Appl. Microbiol.*, 106(5): 1629-1639.
- Etebu, E. and A.M. Osborn, 2010. Molecular quantification of the pea footrot disease pathogen (*Nectria haematococca*) in agricultural soils. *Phytoparasitica*, 38: 447-454. DOI: 10.1007/s12600-010-0122-8.
- Felsenstein, J., 1989. PHYLIP-Phylogeny inference package. *Clad.* 5: 164-166.
- Goud, J.C. and A.J. Termorshuizen, 2003. Quality of methods to quantify microsclerotia of *Verticillium dahliae* in soil. *Euro. J. Plant Pathol.*, 109: 523-534.
- Hagedorn, D.J., 1991. *Handbook of Pea Diseases*, Cooperative Extension Publications, University of Wisconsin, Madison, USA.
- Hall, T.A., 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, NuCl. *Acids Symp. Ser.*, 41: 95-98.
- Han, Y.N., X.G. Liu, U. Benny, H.C. Kistler and H.D. Van-Etten, 2001. Genes determining pathogenicity to pea are clustered on a supernumerary chromosome in the fungal plant pathogen *Nectria haematococca*. *Plant J.*, 25: 305-314.
- Hobbs, S.L.A. and J.D. Mahon, 1982. Variation, heritability, and relationship to yield of physiological characters in peas. *Crop Sci.*, 22: 773-779.
- Idnurm, A. and B.J. Howlett, 2001. Pathogenicity genes of phytopathogenic fungi. *Mol. Plant Pathol.* 2: 241-255.
- Johnsen, K., O. Enger, C.S. Jacobsen, L. Thirup and V. Torsvik, 1999. Quantitative selective PCR of 16S ribosomal DNA correlates well with selective agar plating in describing population dynamics of indigenous *Pseudomonas* spp. in soil hot spots. *Appl. Environ. Microbiol.*, 65: 1786-1789.
- Kabir, S., N. Rajendran, T. Amemiya and K. Itoh, 2003. Quantitative measurement of fungal DNA extracted by three different methods using real-time polymerase chain reaction. *J. Biosci. Bioeng.*, 96: 337-343.
- Kraft, J.M., 1984. *Fusarium Root Rot*. In: Hagedorn, D.J. (Ed.), *Compendium Pea Disease*. American Phytopathological Society, St. Paul, MN, USA, pp:30-31.
- Lechner, S. and R. Conrad, 1997. In soil aerobic hydrogen-oxidising bacteria related to (*Alcaligenes eutrophus*) by PCR and hybridization assays targeting the gene of the membrane-bound (NiFe) hydrogenase. *FEMS Microbiol. Ecol.*, 22: 193-206.
- Mackintosh, S.F., D.E. Matthews and H.D. Vanetten, 1989. Additional genes for pisatin demethylation and their relationship to the pathogenicity of *Nectria haematococca* on pea. *Mol. Plant-Microbe Interact.* 2: 354-362.

- Mahuku, G.S. and H.W. Platt, 2002. Quantifying *Verticillium dahliae* in soils collected from potato fields using a competitive PCR assay. *Am. J. Pot. Res.*, 79: 107-117.
- Mahuku, G.S., H.W. Platt and P. Maxwell, 1999. Comparison of polymerase chain reaction methods with plating on media to detect and identify *Verticillium* wilt pathogens on potato. *Can. J. Plant Pathol.*, 21: 125-131.
- Marino, J.H., P. Cook and K.S. Miller, 2003. Accurate and statistically verified quantification of relative RNA abundances using SYBR Green I and real-time RT PCR. *J. Immunol. Methods*, 283: 291-306.
- Matthews, D.E. and H.D. Van-Etten, 1983. Detoxification of the phytoalexin pisatin by a fungal cytochrome P450. *Arch. Biochem. Biophys.*, 224: 494-505.
- McPhee, K., 2003. Dry pea production and breeding-a mini-review. *Food Agric. Environ.*, 1: 64-69.
- Miao, V.P.W., S.F. Covert and H.D. Van-Etten, 1991a. A fungal gene for antibiotic resistance on a dispensable ("B") chromosome. *Science*, 254: 1773-1776.
- Miao, V.P.W., D.E. Matthews and H.D. Van-Etten, 1991b. Identification and chromosomal locations of a family of cytochrome-P-450 genes for pisatin detoxification in the fungus *Nectria haematococca*. *Mol. Gen. Genet.*, 226: 214-223.
- Navas-Cortés, J.A., A.R. Alcalá-Jiménez, B. Hau and R.M. Jiménez-Díaz, 2000. Influence of inoculum density of races 0 and 5 of *Fusarium oxysporum* f. sp. *ciceris* on development of Fusarium wilt in chickpea cultivars. *Eur. J. Plant Pathol.*, 106: 135-146.
- Nicholas, K.B. and H.B. Nicholas, 1997. GeneDoc: A tool for editing and annotating multiple sequence alignments. Retrieved from: <http://www.psc.edu/biomed/genedoc>.
- Oyarzun, P.J., 1993. Bioassay to assess root rot in pea and effect of root rot on yield. *Neth. J. Plant Pathol.*, 99: 61-75.
- Rush, C.M. and J.M. Kraft, 1986. Effects of inoculum density and placement on fusarium root rot of peas. *Phytopathology*, 76: 1325-1329.
- Smith, C.J., D.B. Nedwell, L.F. Dong and A.M. Osborn, 2006. Evaluation of quantitative polymerase chain reaction-based approaches for determining gene copy and gene transcript numbers in environmental samples. *Environ. Microbiol.*, 8: 804-815.
- Sugha, S.K., S.K. Kapoor and B.M. Singh, 1994. Factors influencing fusarium wilt of chickpea (*Cicer arietinum* L.), India *J. Mycol. Pl. Pathol.*, 24: 97-102.
- Temporini, E.D. and H.D. Van-Etten, 2002. Distribution of the pea pathogenicity (PEP) genes in the fungus *Nectria haematococca* mating population VI. *Curr. Genet.* 41: 107-114.
- Van-Etten, H.D., D.E. Matthews and P.S. Matthews, 1989. Phytoalexin detoxification: Importance for pathogenicity and practical implications. *Annu. Rev. Phytopathol.*, 27: 143-164.
- Van-Etten, H., E. Temporini and C. Wasmann, 2001. Phytoalexin (and phytoanticipin) tolerance as a virulence trait: Why is it not required by all pathogens? *Physiol. Mol. Plant Pathol.*, 59: 83-93.
- Wasmann, C.C. and H.D. Van-Etten, 1996. Transformation-mediated chromosome loss and disruption of a gene for pisatin demethylase decrease the virulence of *Nectria haematococca* on Pea. *Mol. Plant-Microbe Interact.*, 9: 793-803.