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The epidemiology of rhizomania in the Pithiviers region of France: diversity, micro-evolution and interaction with cultivars at the field scale

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ABBREVIATIONS

aa	Amino acid
ABP1	Auxin binding protein 1 gene
Avr	Avirulence
BBSV	*Beet black scorch virus*
BLAST	Basic local alignment search tool
BNYVV	*Beet necrotic yellow vein virus*
bp	base pairs
BSBMV	*Beet soil-borne mosaic virus*
BSBV	*Beet soil-borne virus*
BVQ	*Beet virus Q*
cDNA	complementary DNA
CP	Coat Protein
CP-RT	Coat Protein, Readthrough
Cq	Quantification cycle
CRG	Centre for Genomic Regulation
C. quinoa	*Chenopodium quinoa*

DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DPI	Days post-inoculation
HEL	Helicase
H. schachtii	*Heterodera schachtii*
HKG	Housekeeping gene
IAA	Indole-3-acetic acid
IPC	Internal positive control
ITB	Institut technique français de recherche sur la betterave
ITS	Internal transcribed spacers
IU/gr soil	Infectious unit per gram of soil
kDa	kiloDalton
MIQE	Minimum Information for publication of Quantitative real-time PCR Experiments
MPN	Most Probable Number
mRNA	messenger RNA
MTR	Methyl-transferase
NCBI	National Center for Biotechnology Information
NES	Nuclear exportation signal
NGS	Next generation sequencing
NLS	Nuclear localization signal
ORFs	Open Reading Frame
Pb	*Polymyxa betae*
PCR	Polymerase chain reaction
PRO	Protease

PSD	Supply and distribution online database
PTGS	Post-transcriptional gene silencing
PMTV	*Potato mop-top virus*
qRT-PCR	Real-time quantitative reverse transcription PCR
RACE	Rapid amplification of polymorphic DNA PCR
RAPD	Random amplification of polymorphic DNA
RDML	Real-time PCR Data Markup Language
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNAi	RNA interference
rRNA	ribosomal RNA
siRNA	small interfering RNA
SNP	Single-Nucleotide Polymorphism
SSCP	Single-Strand Conformation Polymorphism
TGB	Triple gene block
UFS	Union Française des Semenciers
USDA	United States Department of Agriculture
UTR	Untranslated region

GENERAL INTRODUCTION

Rhizomania in sugar beet

Rhizomania is the most important viral disease on sugar beet (*Beta vulgaris*). The first detection of rhizomania in France was reported in 1974 and in 1984 in the USA (Duffus, 1984). Rhizomania sometimes reduce the yield up to 70% in fields strongly affected by the disease (Putz *et al.*, 1990; Whitney and Duffus, 1991; Rush and Heidel 1995). The disease causes mainly root symptoms and sporadic leaf symptoms. Affected by the disease, the taproot decreases in volume. There is atypical formation of secondary rootlets (Tamada *et al.*, 1999). The root and foliar symptoms are presented in figure 1.

The rhizomania disease was discovered in 1952. It has been documented for the first time in 1960's in the region of Po in Italy (Canova, 1959). Keskin described in 1964 the new parasite or more precisely the endoparasite *Polymyxa betae*. The responsible agent for the rhizomania disease is the *Beet necrotic yellow vein virus* (BNYVV) identified for the first time by Tamada *et al.* in 1971. For the moment, this agent is classified as quarantine pest in the A2 list of Europe (locally present in the EPPO region (European and Mediterranean Plant Protection Organization)). The transmission of the virus by the vector *Polymyxa betae* was highlighted by Fujisawa and Sugimoto in 1977. Because the disease is caused by a virus, there is currently no real possibility for the farmers to control the disease with phytopharmaceutical products or chemicals. Alternative, environmentally-friendly methods, safe for consumers, have been proposed against zoosporic vectors, such as the use of composite-like azadirachtin (Akça *et al.*, 2005) or salicylic acid (Burketovà *et al.*, 1996) or microorganisms like *Pseudomonas fluorescens* (Kristek *et al.*, 2010), *P. putida* (Aksoy and Yilmaz, 2008), *Streptomyces* sp. (Wang *et al.*, 2003), or *Trichoderma* sp. (Yilmaz and Tunali, 2010), either in soil mixtures or as seed dressings, but their efficiency in reducing BNYVV soil inoculum for rhizomania control on a large scale is not clear. The only solution is the natural resistance of sugar

beet plants (by introgressive hybridization with *Beta maritima* and *Beta vulgaris*) (Stark *et al.*, 2006) or the use of transgenic plants (Lennefors *et al.*, 2008; Pavli *et al.*, 2010).

Fig. 1. Rhizomania symptoms. All the pictures have been taken around the Pithiviers area. Foliar symptoms: a. Systemic foliar symptoms with yellowing and necrosis of leaves veins; b. Foliar wilt of infected sugar beets; c. Yellowing leaves of rows of susceptible sugar beets (variety 'Encarta'). Root symptoms: d. Vascular system browning and lignification (vb); e. Proliferation of lateral rootlets (beard-like appearance of taproot); f. Root constriction (rc); g. Reduction of the taproot size of *Rz1* cultivars with weak resistance (right) in comparison with *Rz1* cultivars with good resistance (left).

In France, it is already a long time ago that there is a monitoring of the rhizomania disease in fields. The first resistance-breaking events appeared in classical varieties in the 70's in France and BNYVV was identified (Tamada and Baba, 1973). Genetic drift models for the evolution of BNYVV

(where aa changes would not evolve significantly faster than neutrality) were rejected in favour of models including positive, diversifying selection (Schirmer *et al.*, 2005). It is in 2002, that resistance-break appeared again in the *Rz1* varieties (Tamada *et al.*, 2003; Rush and Acosta-Leal, 2007; Liu and Lewellen, 2007). One of the specifications of this pathosystem is also the long term survival of the virus and the vector in the soil, through the *Polymyxa* resting spores.

Polymyxa betae, the vector of BNYVV

The BNYVV is transmitted by the vector *Polymyxa betae*. The vector is an obligate intra-cellular parasite which develops itself in the epidermic and cortical cells of beet roots. *P. betae* is a protist which belongs to the *Plasmodiophoridae* (Archibald and Keeling, 2004) (Table 1). The vector is found in the entire beet areas (Rush, 2003). The vector is also found in other host species as *Brassicaceae*, *Papaveraceae*, *Poaceae* and *Urticaceae* (Legrève *et al.*, 1998; Legrève *et al.*, 2000; Legrève *et al.*, 2005).

Table 1. Classification and taxonomy of *Polymyxa betae* (Encyclopedy of Life).

Polymyxa betae Scientific classification	
Domain:	Eucaryote
Kingdom:	Rhizaria (Protozoa)
Phylum:	Cercozoa
Subphylum:	Endomyxa
Class:	Plasmodiophorea (Phytomyxea)
Order:	Plasmodiophorida
Family:	Plasmodiophoridae
Genus:	Polymyxa
Species:	*P. betae K*eskin 1964

All the viruses transmitted by *P. betae* are interiorized (Rysanek *et al.*, 1992; Campbell, 1996 and Verchot-Lubicz *et al.,* 2007) in the cytoplasm of this vector (circular transmission (Hébrard *et al.*, 1999)) and consequently, the biological cycle of the virus diseases are dependent of the vector life cycle (Figure 2).Verchot-Lubicz *et al.* (2007) describes *Polymyxa betae* as a possible host for BNYVV. BNYVV is carried internally by approximately 10% to 20% of *P. betae* resting spores in the soil (Tuitert, 1990; Mc Grann *et al.*, 2009). It is generally accepted that BNYVV does not replicate inside the vector, but recently evidence from Verchot-Lubicz *et al.* (2007) suggests that this may not necessarily be the case (Mc Grann *et al.*, 2009).
It is supposed that the virus transmission occurs through the sporogenic and sporangial plasmodia. The RT domain of BNYVV plays an important role in the transmission by the vector. The substitution of the four amino acids $_{553}$KTER$_{556}$ of the RT domain by the ATAR motif completely prevents the transmission (Tamada *et al.*, 1996). This vector has also a long term survival

capacity. Its thick-walled sporosores can survive during many years in the soil and it can be disseminated largely (Adams, 1990; Maraite, 1991; Wisler and Duffus, 2000). The consequence of this survival is the virus disease recurrence. The optimum condition for the vector are firstly the host presence, secondly the presence of free water in the soil and thirdly a optimum temperature comprised between 10 and 30 °C with an optima between 20 and 25 °C according to the different isolates (Legrève *et al.*, 2002).

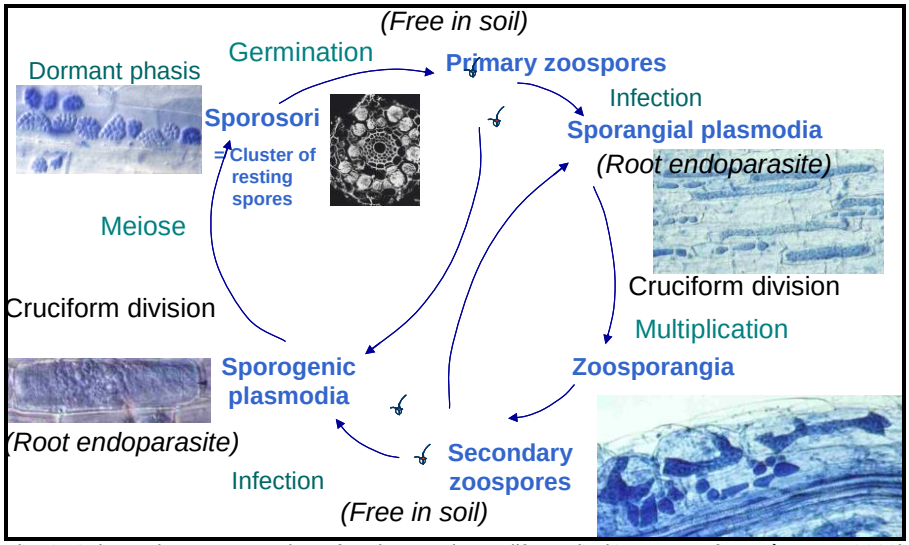

Fig. 2. Schematic representation of *Polymyxa betae* life cycle (Courtesy of Legrève, A.; 2002). The different development steps are the following: 1) sporosori (5-7µm) which is a mass of resting spores; 2) primary biflagellate zoospores (4-5 µm) germination; 3) infection with zoospore swimming to the epidermic or cortical root cell; 4) zoospore encystment on this cell and injection of the cytoplasm and nucleus of the zoospore through the cell wall and cell membrane with the help of the Stächel (needle-like structure) (3h after inoculation); 5) sporangial plasmodia (virus transmission; 24h after inoculation for the beginning of the plasmodial stage and five days after inoculation for the plasmodial stage) development evolving in zoosporangia in the sporangial phasis; 6) zoosporangia releasing secondary zoospores able to reinfect a new cell (15 days after inoculation) or able to evolved in sporogenic plasmodia in the sporogenic phasis; 7) sporogenic plasmodia (virus transmission; 15 days after inoculation) producing sporosori liberated in the soil during the root tissues decomposition.

BNYVV, the causal agent of rhizomania

The causal agent of rhizomania is *Beet necrotic yellow vein virus* (BNYVV), a *Benyvirus* (Torrance and Mayo, 1997). The virus is multipartite and is composed of four or five rod-shaped particles of 20 nm diameter (Putz, 1977; Tamada *et al.*, 1989). BNYVV is composed of four or five positive single strand RNAs (Figure 3) (Hleibieh *et al.*, 2007). RNAs are capped at the 5' UTR (m^7GpppG) and polyadenylated in 3' UTR for a length of 65-140 nucleotides (Putz *et al.*, 1983). Several virus pathotypes, serologically identical, have been detected and classified according to RFLP and SSCP of RT-PCR products (Kruse *et al.*, 1994; Koenig *et al.*, 1995b; Mehrvar *et al.*, 2009). A pathotype is an infrasubspecific classification of a pathogen

distinguished from others of the species by its pathogenicity on a specific host(s), characterized by its pattern of virulence or avirulence to a series of differential host varieties. The nucleotide variation inside a pathotype is usually limited. Four pathotypes are known: the A-pathotype, B-pathotype, P-pathotype and J-pathotype, geographically located (Schirmer *et al.*, 2005). The A- and B-pathotype are composed of four RNAs while the P- (Pithiviers, France) and J-pathotype are often composed of five RNAs (4,2% of difference between the J-pathotype and the P-pathopathotype for the nucleotide sequence) (Koenig *et al.*, 1997b). But, there are also viruses in the P-pathotype which have not the fifth RNA. The viruses with a fifth RNA are known to be more aggressive on sugar beet (Tamada *et al.*, 1997). The RNA5 is divided in three groups: the first one is composed of Chinese isolates (Li *et al.*, 1999), the second of Japanese isolates (Myianishi *et al.*, 1999) and the third of Kazakhstan (Koenig and Lennefors, 2000) and French isolates (Koenig *et al.*, 1997b).

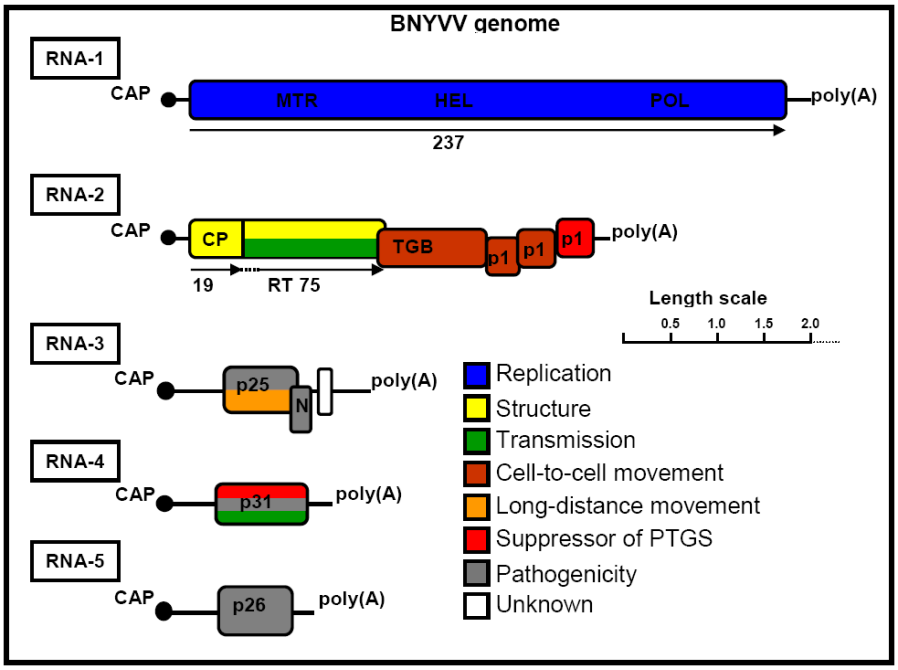

Fig. 3. Schematic representation of BNYVV genome organization (François Crutzen *et al.*, 2008)

BNYVV RNA1 possesses only one ORF and encodes for a polyprotein (replicase) of 237 kDa autocatalytic cleaved. The RNA is coding for methyl-transferase (MTR), helicase (HEL), protease (PRO) and RNA-dependent-RNA polymerase (POL) domains. The role of theses proteins are involved in virus replication (Bouzoubaa *et al.*, 1987). The multicistronic RNA2 contains several ORFs (six) (Bouzoubaa *et al.*, 1986; Putz, 1977; Richards *et al.*, 1985; Schmitt *et al.*, 1992 and Gilmer *et al.*, 1992). The two first ORFs are coding for coat-protein (CP) of 21 kDa and a coat-protein readthrough (CP-RT) of 75 kDa (for 10% of translation events). The CP is responsible of the

virus structure and the CP-RT is responsible of the virus structure and virus transmission. The others ORFs of subgenomic RNAs encodes for proteins of the Triple Gene Block (TGB), TGB-p1 of 42 kDa, TGB-p2 of 13 kDa and TGB-p3 of 15 kDa (Erhardt *et al.*, 2005). These proteins are involved in the cell-to-cell movement. The sixth ORF of the RNA2 encodes for a protein p14 of 14 kDa which is responsible of the suppression of the plant post-transcriptional gene silencing (PTGS) (Tamada, 1999; Dunoyer, *et al.* 2002). The RNA3 encodes for three ORFs. The first gene is coding for the protein p25 (25 kDa) and is responsible for the virus pathogenicity. The second one encodes for the protein N (6,8 kDa) and is responsible for the pathogenicity and long distance movement (LDM). The last ORF is p4,6 (4,6 kDa) and its function is unknown (Bouzoubaa *et al.*, 1985; Tamada *et al.*, 1989 and 1999; Jupin *et al.*, 1992). The RNA4 encodes for the protein p31 (31 kDa) and is responsible of virus transmission by the vector (Lemaire *et al.*, 1988), PTGS suppressor in sugar roots (Rahim *et al.*, 2007) and also pathogenicity (Bouzoubaa *et al.*, 1985; Tamada *et al.*, 1989; Chenggui *et al.*, 2002; Rahim *et al.*, 2007). The fifth RNA, known to exacerbate the aggressivity of BNYVV encodes for the protein p26 (26 kDa) and is also involved in the pathogenicity (Link *et al.*, 2005). This RNA has only been found in Pithiviers (France) (Koenig *et al.*, 1997b), Kazakhstan (Koenig and Lennefors, 2000), UK (Harju *et al.*, 2002; Ward *et al*., 2007)), Asia as China (Koenig and Lennefors, 2000) and Japan (Tamada *et al*., 1989; Tamada *et al.*, 1996; Tamada *et al*., 1989; Kiguchi *et al*., 1996; Miyanishi *et al*., 1999), and more recently in Germany (Koenig *et al*., 2008). Like p25, p26 is actively transported in the nucleus of plant cells. It was demonstrated also that p26 acts in synergism with p25 with regard to the symptom severity (Kiguchi *et al.*, 1996; Link *et al.*, 2005).

RNAs of BNYVV in relation with the plant

The following lines will focus on three different RNAs (RNA3, RNA2 and RNA5). The RNA3 is responsible for the virus propagation and multiplication in the sugar beet roots (Tamada and Abe, 1989). The viral contents in the root level, in the presence of the third RNA, are higher than in its absence (Koenig *et al.,* 1991). The RNA3 encodes for a pathogenicity factor p25 (220 amino acids). BNYVV RNA3 is responsible for symptom expression and yield reduction (Koenig *et al.*, 1991). P25 is involved in virus translocation from lateral roots into the taproot in susceptible cutlivars (Tamada *et al.*, 1999). The nucleotide region 1033 to 1257 in RNA3 (more or less the nucleic region of N gene) is involved in the vascular movement (systemic) in the host *Beta macrocarpa* (Lauber *et al.*, 1998b). P25 is able to shuttle between the nucleus and the cytoplasm of infected cells (Haeberlé and Stussi-Garaud, 1995; Vetter *et al.*, 2004). The four valine residues in positions 169, 172, 175 and 178 are all necessary for nucleocytoplasmic shuttling. Mutants that lack this shuttling ability fail to produce yellow lesions on *C. quinoa* (Koenig, 2008). The presence of the p25 in the nucleus is necessary for disease symptom expression. The protein p25 contains a NLS (Nuclear localization signal) in the aa. 57-62 with a **KR**IRF**R** pattern and a NES (Nuclear exportation signal: **V**YM**V**CL**V**NT**V**) highly conserved in the aa

169-178 (Figure 4). These two sequences, allowing to p25 a nucleocytoplasmic shuttling, are dependent of alpha importin and exportin 1 but are independent of the expression of others viral proteins (Vetter *et al.*, 2004). P25 is highly variable at aa positions 67-70 (nucleic acid position 199-210) (Schirmer *et al.*, 2005). These four amino acids are called tetrad. A connection between, the composition of this tetrad and the severity of symptoms in beets has been discussed, but not yet proven. In *Tetragonia expansa*, the tetrad pattern influences the foliar symptom severity (Klein *et al.*, 2007). The severe necrotic lesions were observed for AYHR tetrad, yellow lesions for SYHG, AYRV and AFHR, green chlorotic lesions for AHHG, ALHG and AFHG, and finally, very weak chlorotic lesions for VCHG, ACHG and VLHG. BNYVV p25 tetrad mutants possess resistance breaking abilities (Liu *et al.*, 2005 and 2007; Acosta-Leal *et al.*, 2007a and 2008; Pferdmenges *et al.*, 2008 and 2009a). P25 tetrad VCHG (aa 67-70) is responsible for *Rz1* resistance-breaking in USA (Koenig *et al.*, 2009b). Acostal-Leal and Rush (2007a) determined two mutations in p25 gene coding for the virus pathogenicity protein. These mutations are A/V_{67} in the hypervariable tetrad and D/E_{135} in the C-terminal region of the protein. In 2008, the position 68 in the tetrad was evaluated highly variable between resistance breaking strains and wild-type isolates. Klein *et al.* (2007) have localized a transcriptional activation domain for the gene His3 in a "yeast-one-bybrid system. P25 has also a zinc-finger motif in aa positions 73–90 which is usefull for DNA fixation and there is an acid (C-terminal) and basic (N-terminal) region in the p25. The protein is able to multimerize and the interaction between the different p25 proteins requires the integrity of p25 to produce yellow chlorotic lesions in the host (Klein *et al.*, 2007).

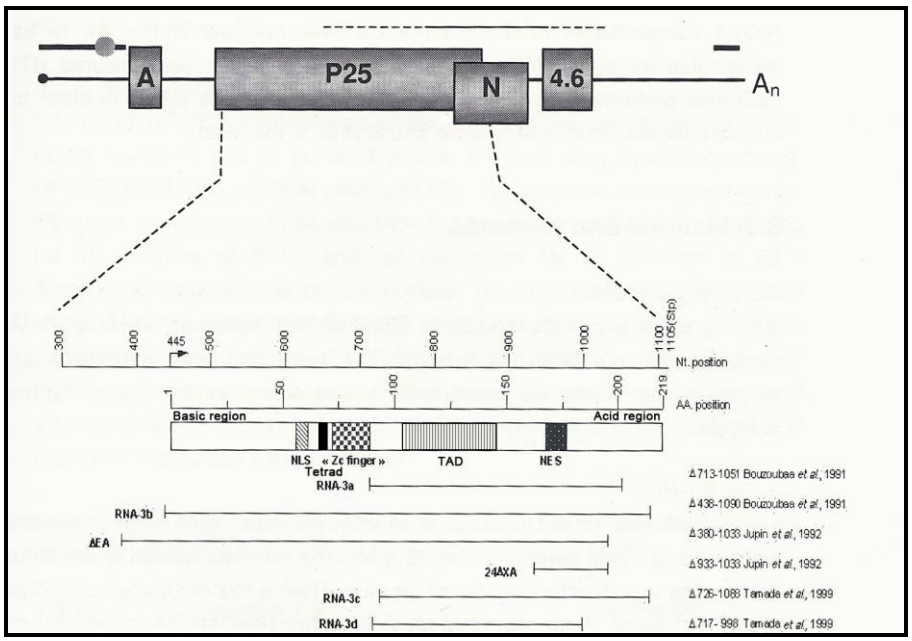

Fig. 4. Schematic representation of p25 protein organization (Modified schedule from Meunier, A., 2005). The box symbolizes p25 ORF. The localisation of the "zinc-finger" motif on RNA3 is

symbolised by the spotted box ("Zc Finger": aa 73-90) and the "Nuclear localization signal" (NLS: 57**KR**IRF**R**62) and the "Nuclear exportation signal" (NES: 169**V**YM**V**CL**V**NT**V**178) are indicated. The hypervariable region called tetrad (aa 67-70), the transcription activation domain (aa 103-146), the basic and acid region is also indicated. The thick grey lines above the RNA molecule at the 3' and 54 extemities indicate the secondary structure involved in the replication process and the grey dot indicate the encapsidation sequence. The narrow grey line above p25 ORF represents sequences involved in the subgenomic RNA synthesis. The lines located under the box represent deletions occurring in RNA3 molecule and described in the literature.

The small N gene is not detectably expressed from full-length RNA3, but it is translationally activated by the deletion of upstream sequences which positions it closer to the 5' end of RNA3 (Jupin *et al.*, 1992). Expression of the N gene induces the formation of necrotic lesions in BNYVV infections and also when it is expressed by another plant virus like *Cauliflower mosaic virus* (Koenig, 2008). Chiba *et al.* (2008) discussed the role of p25 protein as an avirulence factor (dominant gene) and a pathogenicity factor (recessive gene). They showed that p25 is responsible for the immunity response activation in the plant. The protein induces symptoms formation of the disease and acts as a inducer of the plant resistance. Schmidlin *et al.* (2008) showed that there is differential expression of cellular genes in BNYVV infected *Beta vulgaris*. In 2009, Koenig *et al.* and Acosta-Leal *et al.* showed that the resistance *Rz1* breakdown in USA was essentially due to an aa change in position 67. In 2010, Acosta-Leal et al. confirms the breakdown of sugar beet resistance by p25 pathogenicity factor. Peltier *et al.* (2011) showed that p25 induces root branching in transgenic *Arabidopsis thaliana*. The function of the tetrad in the p25 is still not known but recently the p25 pathogenicity factor of *Beet necrotic yellow vein virus* was supposed to target the sugar beet 26S proteasome involved in the induction of a hypersensitive resistance response via interaction with an F-box protein (FBK). Thiel *et al.* confirmed this sugar beet FBK-p25 interaction *in vivo* and *in vitro* and provided evidence for in planta interaction and similar subcellular distribution in *Nicotiana tabacum* leaf cells. The results support the idea that p25 is involved in virus pathogenicity in sugar beet and suggest suppression of resistance response (Thiel *et al.*, 2012). These F-box proteins are one of three components of the SCF complex which catalyzes the ubiquitination of proteins destined for proteosomal degradation. They have also been associated with cellular functions such as signal transduction and regulation of the cell cycle (Craig and Tyers, 1999). In plants, many F-box proteins are represented in gene networks broadly regulated by microRNA-mediated gene silencing via RNA interference (Jones-Rhoades *et al.*, 2006).

The RNA5 enhances symptom expression in sugar beet. Scab-like symptoms rather than root proliferation have been observed with a recombinant virus containing RNA5, but lacking RNA3. Addition of P-pathotype RNA5 to RNA1 and -2 results in the formation of necrotic local lesions in *C. quinoa* (Link *et al.*, 2005; Schmidlin *et al.*, 2005).The RNA5 encoded p26 apparently occurs mainly in the nucleus (the transport inside the nucleus is active) as suggested by studies with fluorescent fusion proteins (Koenig, 2008). This fifth RNA is present with some viral isolates and induces very severe rhizomania symptoms (Hauser *et al.*, 2000). Consequently, this RNA has a huge incidence on the sugar yield (Heijbroek

et al., 1999). More recently, new variants of BNYVV have evolved (both with and without RNA5) that are able to cause significant yield penalties on resistant cultivars (Mc Grann *et al*., 2009). The p26 activates strongly the transcription in the yeast simple hybrid system (Link *et al.*, 2005). The only sequence homology between p25 and p26 is $Fx_3FRGPGNx_2L$ just after the transcriptional activation domain. P25 and p26 act in synergism for the symptom appearance. Two distinct BNYVV RNA5s occurred in UK in 2005 (Ward *et al.*, 2005).

The RNA2 encodes for the p14 cystein-rich protein, a putative RNA silencing suppressor (Dunoyer *et al.*, 2002). This protein is expressed from the subgenomic RNA 2sub-c. This protein plays also a role in the viral replication regulation through the stimulation of the accumulation of the capsid protein (Gilmer *et al.*, 1992; Hehn *et al.*, 1995; Hleibieh *et al.*, 2007). Gilmer *et al.* (1992) observed a decrease of foliar necrotic lesions diameter on sugar beet in the absence of p14. The concentration of the others RNAs diminished of 10 until 30 times.

P25 and auxin

BNYVV infected susceptible sugar beets showing heavy lateral root growth and increase of free auxin (Poggi Pollini *et al.*, 1990). Expression analysis of susceptible sugar beet genotypes infected with BNYVV identifying pathogen induced proteins. There are effects on many phytohormones and stress proteins, including auxin-related proteins (Larson *et al.*, 2008). Schmidlin *et al.* (2008) identified virus induced expression of auxin binding protein 1 gene (ABP1). Peltier *et al.* (2011) showed that p25 transgenic *Arabidopsis thaliana* plants were more susceptible than wild-type plants to auxin analog treatment (2,4-D). Auxin (Auxin/Indole-3-acetic acid (IAA)) is responsible of cell division, extension and differentiation in the cellular level of the sugar plant and is responsible for apical dominance, lateral. Adventitious root formation, tropism, fruit set and development, vascular differentiation as embryogenesis in the whole plant level. Theses effects are the symptoms observed for the rhizomania disease. Aux/IAA proteins are short lived transcription factors that share four highly conserved domains. And within these proteins, there is a region where there is no polymorphism between Bv-IAA amplified from resistant (*Rz1, Rz2*) and susceptible sugar beet lines (Thiel *et al.*, 2009 and 2011). This Bv-IAA interacts *in vivo* (Yeast-two hybrid (YTH)), *in planta* (Bimolecular fluorescence complementation (BiFC)) and *in vitro* (glutathione S-transferase (GST-pull down)) with BNYVV p25 and is localized in the nucleus of *Nicotiana benthamiana*. Bv-IAA changes its localization when it is co-expressed with p25. The interaction p25-Bv-IAA is auxin inducible (Thiel *et al.*, 2011).

Benyvirus, Pomovirus and *Necrovirus*

If BNYVV is the causal agent of rhizomania on sugar beet, this virus is often associated with several RNA viruses, transmitted also by *P. betae*. In the *Benyvirus*, there is also *Beet soil-borne mosaic virus* (BSBMV) (Liu and

Duffus, 1988; Mahmood and Rush, 1999; Workneh *et al.*, 2003; Ratti *et al.*, 2009). BSBMV is only present in North America. In the USA, BSBMV RNA3 complements BNYVV RNA3 and is replicated and encapsidated by BNYVV (Ratti *et al.*, 2009). There are also two *Pomovirus* transmitted by this vector, *Beet-soil-borne virus* (BSBV) and *Beet virus Q* (BVQ), which are present in Europe (Crutzen *et al.*, 2009 and 2010). There is another RNA virus detected on sugar beet in China and USA named *Beet black scorch virus* (BBSV) transmitted by *Olpidium brassicae* (Russo *et al.*, 1981; Liu and Xian, 1995; Hleibieh *et al.*, 2007; Weiland *et al.*, 2007; Mehrvar and Bragard., 2008).

The temperature has an effect on the virus replication rate in the plant. The optimum conditions for the vector *Polymyxa betae* are 10/30 °C with an optimum at 20/25 °C (Legrève *et al.*, 2002). In comparison, the optimum conditions for the viruses are the following: for BNYVV, the optimum temperature is 20/25 °C (virus already detected in plants after two weeks of culture) (Goffart and Maraite, 1992); for BSBV, the optimum temperature for the higher rate of synthesis of BSBV-2 is below 20 °C (Prillwitz and Schlösser, 1993). The temperature has an effect on the virus replication rate in the plant. BSBV is the virus which is developing the faster. Consequently, in the sugar beet season, the BSBV infects the plant firstly in spring and the infection of BNYVV increases in July when the temperature is higher. Moreover, the preinfection of sugar beets with BSBV/BVQ reduced significantly the BNYVV content but increased significantly the number of cystosori (Prillwitz and Schlösser, 1992). It seems that there is firstly a competition between different viruses early in the season. BVQ is almost systematically present if BSBV or BNYVV is present (Meunier *et al.*, 2003) and almost never alone (in the PCR detection threshold). However, through mechanical inoculation of full-length infectious clones, there is no rescue of BVQ by BSBV but there is a rescue of BVQ by complementation with BNYVV (Crutzen and Bragard, 2010). Sometimes, BSBV/BVQ and BNYVV are in competition. When one increases, the other one decreases (Prillwitz and Schlösser, 1993). Either BSBV or BNYVV infecting in first time avoids the overinfection of the second in the same sugar root. There is consequently an effect of crossprotection. This is the case for BSBMV and BNYVV through a repression of structural proteins through one of the two viruses (Mahmood and Rush, 1999; Workneh *et al.*, 2003).

Benyvirus: BNYVV and BSBMV

The two viruses in sugar beet which belonging to the *Benyviruses* are BNYVV and BSBMV. The BNYVV has been fully described above. *Beet soilborne mosaic virus* (BSBMV) is a rigid rod-shaped virus transmitted by *Polymyxa betae*. This virus is only reported in USA (Wisler *et al.*, 1994; Ratti *et al.*, 2009). Particles were 19 nm wide and ranged from 50 to over 400 nm. Nucleic acids extracted from virions were polyadenylated. RNA1 and -2, the largest of the RNAs, consistently averaged 6,7 and 4,6 kb, respectively. The sizes and number of smaller RNA species were variable. The molecular mass of the capsid protein of BSBMV was estimated to be 22,5 kDa. In Northern blots, probes specific to the 3´ end of individual *Beet necrotic*

yellow vein virus (BNYVV) RNAs 1–4 hybridized strongly with the corresponding BNYVV RNA species and weakly with BSBMV RNAs 1, 2, and 4. Probes specific to the 5´ end of BNYVV RNAs 1–4 hybridized with BNYVV but not with BSBMV. No cross-reaction between BNYVV and BSBMV was detected in Western blots. In greenhouse studies, root weights of BSBMV-infected plants were significantly lower than mock-inoculated controls but greater than root weights from plants infected with BNYVV. Results of serological, hybridization, and virulence experiments indicate that BSBMV is distinct from BNYVV. However, host range, capsid size, and the number, size, and polyadenylation of its RNAs indicate that BSBMV more closely resembles BNYVV than it does other members of the genus *Benyvirus* (Heidel *et al.*, 1997).

Pomovirus: BSBV and BVQ

The two viruses belonging to the *Pomovirus* are BSBV and BVQ. BSBV and BVQ are both transmitted by *Polymyxa betae*. BSBV, BVQ and BNYVV are often found associated in the fields and in mixed infections in sugar beet (Meunier *et al.*, 2003; Ratti *et al.*, 2005). These two viruses are described in the figure 5 (Crutzen and Bragard, 2010).

BSBV has been identified for the first time in England in 1983 by Ivanovic *et al.* The virus is now reported in Sweden and Germany (Lesemann *et al.*, 1989), Finland (Bremer *et al.*, 1990), France, Spain, Italy and The Netherlands (Lindsten, 1993), The USA (Lindsten and Rush, 1994), Belgium (Stas *et al.*, 2001a), Iran (Farzadfar *et al.*, 2002) Syria (Mouhanna *et al.*, 2002) Bulgaria, Hungary, and Turkey, (Meunier *et al.*, 2003), Austria, Croatia, Lithuania, Japan and Switzerland (Ratti *et al.*, 2005), Poland (Borodynko *et al.*, 2006) and China (Wang *et al.*, 2007b). BSBV is organized in a tripartite genome. There is no functional study carried out until now, except for the CP (Koenig *et al.*, 1997a; Borodynko *et al.*, 2009a). There is two presumably transmission-related TM1 and TM2 predicted in the BSBV CP-RT domain (Adams *et al.*, 2001). The BSBV pathogenicity proved to be difficult to determine in the complex rhizomania syndrome. Chlorotic or necrotic spots, rings and ringspots appearing as soon as five days post-inoculation (dpi) were recorded on *Chenopodium* sp. (Crutzen *et al.*, 2009a). There is occasional or even no symptoms on *Beta* and *Spinacia* sp. (Henry *et al.*, 1986). Pre-infection with BSBV reduced significantly the BNYVV content, when this last was inoculated later. Such cross-protection against BNYVV has also been evidenced with the closly related BSBMV (Mahmood and Rush, 1999). When mechanically inoculated to the beet roots (Kaufmann *et al.*, 1993), BSBV has been shown to be responsible for tap-root weight reduction of 20% even if no foliar symptoms were visible.

Earlier, BVQ was described as the Wierthe serotype of *Beet soilborne virus* (BSBV). Now, on the basis of its genomic properties (Koenig *et al*., 1998), BVQ is recognized as a distinct virus species. BVQ is often found in fields where BSBV and BNYVV are present (Rubies Autonell *et al*., 2006). BVQ has been discovered in sugar beet in Belgium (Stas *et al.*, 2001b), Bulgaria,

Germany, Hungary, France, and The Netherlands (Meunier *et al.*, 2003), Iran, (Farzadfar *et al.*, 2005), Italia, Lithuania, Croatia, England (Ratti, *et al.*, 2005), Poland (Borodynko, 2006) and Spain (Rubies Autonell *et al.*, 2006) but not in China (Wang *et al.*, 2007a). The incidence of BVQ may vary from 30% of the tested samples in Belgium (Stas *et al.*, 2001a) to 1,5% in Iranian ones (Farzadfar *et al.*, 2005) and is always lower to that of BSBV, which is reported in 85% and 9,5% of the tested fields for the same countries, respectively. BVQ has been reported to produce lesions that spread along the veins on *C. quinoa* leaves five dpi (Koenig *et al.*, 1998; Rubies Autonell *et al.*, 2006), but mechanical inoculation of beet roots and leaves have been unsuccessful (Koenig *et al.*, 1998). Its involvement in the rhizomania remains a mystery. But, BVQ seems more aggressive than BSBV on *Chenopodium* sp (Bragard, C., personal communication). BVQ and BSBV have a similar genome and have antisera cross-reactivity. BVQ is organized similarly to BSBV, except for the RT domain of the CP (35 kDa) that is much shorter and lets place for two additional ORFs coding for putative proteins of 9 kDa and 18 kDa. The RT domain contains two transmembrane helices, TM1and TM2 required for the viral transmission by the vector (Adams *et al.*, 2001; Crutzen *et al.*, 2009).

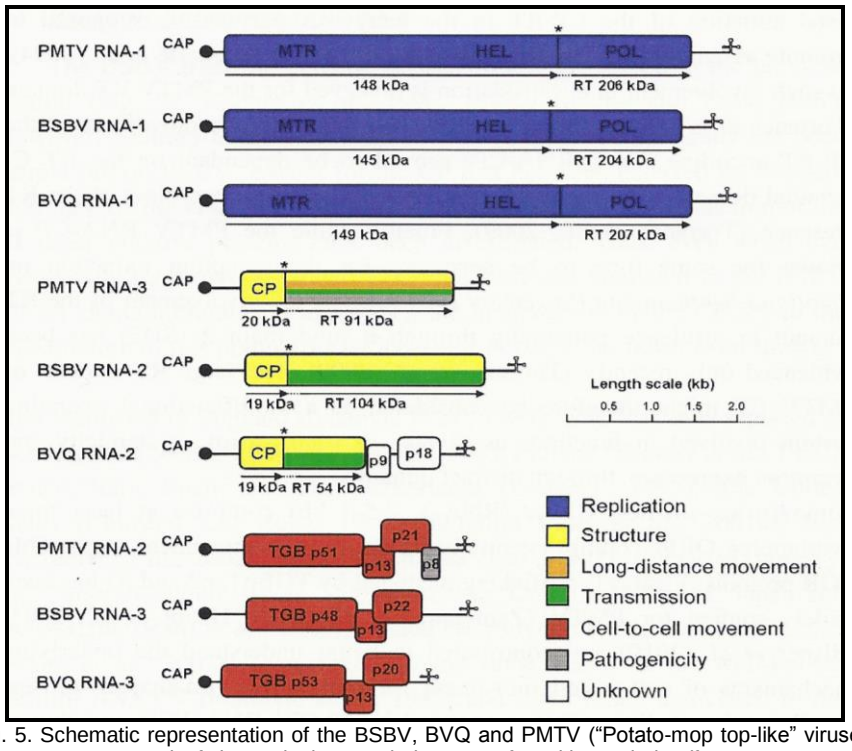

Fig. 5. Schematic representation of the BSBV, BVQ and PMTV ("Potato-mop top-like" viruses) genomes, composed of three single-stranded RNAs of positive polarity (from sequences of Scott *et al.*, 1994; Koenig *et al.*, 1996; Koenig and Loss, 1997; Koenig *et al.*, 1997a; Koenig *et al.*, 1998; Savenkov *et al.*, 1999; Sandgren *et al.*, 2001). 3' ends of all viral RNAs fold in tRNA-like structures whereas 5' endas are thought to be capped. RNAs-1 encoded replicases contain methyl-transferase (MTR), helicase (HEL) and RNA-dependant-RNA polymerase (POL) conserved motifs. The POL motifs are encoded by the smallest RNAs, RNA2 for PMTV and -3

for BSBV and BVQ. PMTV RNA2 comprises also a short cystein-rich protein (CRP) encoding ORF, absent from corresponding RNAs-3 of the other two pomoviruses. Structural coat proteins (CP) and RT domains are expressed from PMTV RNA3 and RNAs-2 of BSBV and BVQ. However, two additional small ORFs encoding for putative proteins of unknown functions are encoded by BVQ RNA2. The molecular mass is indicated in kiloDalton for each protein under or inside the corresponding ORF. Function(s) attributed or suspected for each protein is/are specified through a color code. Leaky termination codons positions for RT domains are indicated by stars (Crutzen and Bragard, 2010).

Necrovirus: BBSV

Beet Black scorch virus (BBSV) was first reported in Inner Mongolia in China in 1988, where it was reported to cause severe black scorch symptoms on the leaves and necrosis in the roots of sugar beet, but no obvious root beardedness. Recently, an isolate of BBSV with deviating molecular and possibly also pathogenic properties has been obtained in the USA from sugar beet plants showing severe rhizomania-like symptoms (root beardedness), but no black scorch symptoms (Koenig and Valizadeh, 2008). Many of these plants were tested negative for BNYVV (Weiland *et al.*, 2006 and 2007). An isolate of BBSV was obtained in 2008 from Iran in a sugar beet roots. Its genome organization closely resembles that of the previously described Chinese and North American isolates, but the nucleotide sequences of the three isolates differ considerably. Most of the nucleotide exchanges, however, are silent, and the Iranian and the Chinese isolates were serologically indistinguishable. Beets infected by the Iranian BBSV (Mehrvar, 2009) did not show black scorch symptoms but severe root beardedness (Mehrvar and Bragard, 2008). This might have been caused by BBSV or the simultaneously present BNYVV, or both together (Koenig and Valizadeh, 2008). A first report of BBSV occurred also in field grown sugar beets with symptoms of rhizomania disease in European country as Spain in 2009 (Gonzalez-Vasquez *et al.*, 2009). Sequences were highly similar to those reported for Chinese and U.S. isolates. Sequence comparisons revealed three clusters of sequences. BBSV was detected in a number of samples with rhizomania symptoms in which BNYVV went undetected. However, its role in rhizomania disease in Europe, if any, remains to be established. BBSV is transmitted in a non-persistent manner by *Olpidium brassicae*. Because of its morphological, biological, and molecular properties, it has been assigned to the genus *Necrovirus* in the family *Tombusviridae* (Cai *et al.*, 1999; Cau *et al.*, 2002; Lommel *et al.*, 2005; Yuan *et al.*, 2006). BBSV transferred to *C. quinoa* caused chlorotic local lesions, at 3 dpi. Symptoms on sugar beet attributed to infection by the virus included a black scorching of leaf tips and severe stunting of affected plants (Cui *et al.*, 1988; Cai *et al.*, 1993). The virus appears to exacerbate symptoms produced by BNYVV. Complete sequence of the positive strand 3,644-nucleotide (nt) genome for two isolates of BBSV from China, 'Xinjiang' (BBSV-X) and 'Ningxia' (BBSV-N), indicates the presence of seven open reading frames (ORFs) organized similarly to those in other *Necroviruses*. Normally, only a single RNA was associated with infections, but a small single-stranded RNA (ssRNA) that is similar to the satellite RNAs (sat-RNA) of other necroviruses (Van Regenmortel *et al.*, 2000) has been found in isolates from the Xinjiang province (Cai *et al.*, 1993). This small RNA

multiplied in test plants only when associated with BBSV genomic RNA, either from the same viral isolate or from other sources of BBSV (Bo *et al.*, 1996).The presence of sat-RNA resulted in more local lesions on the leaves of *Chenopodium amaranticolor* plants than did inoculation with BBSV RNA alone when the inoculum was highly diluted. Many sat-RNAs of plant viruses such as *Turnip crinkle virus* (TCV) (Carpenter *et al.*, 1991), *Cucumber mosaic virus* (CMV) (Kuroda *et al.*, 1997), *Subterranean clover mottle virus* (Davies *et al.*, 1990), and *Lucerne transient streak virus* (Roossinck *et al.*, 1992) have been found to have complicated mechanisms of replication that result in the appearance of different forms of replicating RNAs. Guo *et al.* (2005) reported a sequence analysis of these sat-RNAs and a description of the multimeric forms involved in replication.

Resistance genes in sugar beet

Generally, in host plant, the genes for resistance are dominant (R), whereas genes for susceptibility, i.e., lack of resistance, are recessive (r). In the pathogen, however, genes for avirulence, i.e., inability to infect, are usually dominant (A) whereas genes for virulence are recessive (a). Genes for pathogenecity exist in pathogens against all host plants that lack specific resistance. When a specific gene for resistance appears in or is bred into the host, the gene enables the host to recognize the product of a particular gene for virulence in the pathogen. That pathogen gene is then thought of as the "avirulence" gene (*avr*A) of the pathogen that corresponds to the plant resistance gene R. Subsequent recognition of the *avr*A gene product (the elicitor molecule) by the receptor coded by the R gene triggers the hypersensitive response reaction in the plant that keeps the plant resistant. A new gene for virulence that attacks the existing gene for resistance appears by mutation of an existing avirulence gene, which then avoids gene-for-gene recognition, and the resistance of the host breaks down. Plant breeders then introduce another gene for resistance (R) in the plant, which recognizes the protein of the new gene for virulence of the pathogen and extends the resistance of the host beyond the range of the new gene for virulence in the pathogen. When a variety carries two or more genes for resistance (R1, R2, …) against a particular pathogen, it means that each corresponds to one, two, or more genes of former virulence (and now avirulence) in the pathogen (a1, a2, …), each of which, one recognized by one of the genes for resistance in the host, subsequently functions as an avirulence gene (Agrios, 2004).

There are several plant hosts for the BNYVV: *Beta vulgaris* (systemic infection), *Beta macrocarpa*, wild beet, *Spinacea oleracea*, *Chenopodium quinoa* (necrotic lesions on leaves), *Tetragonia expansa* (chlorotic and necrotic local lesion), *Nicotiana benthamiana* (systemic infection) (Hleibieh *et al.*, 2007). The first classic partial resistant varieties used against the virus were the varieties Rizor (partial resistance in diploid hybrid; SES-italy) and Alba (partial resistant variety from Italy). The variety Alba was introduced in France in 1984. But, the disease was not completely controlled by this variety. The resistance Holly (*Rz1*; Holly Sugar Company) was then

introduced for the first time in 1994 in France and generalized in 2006. The first introduction of the Holly resistance in Germany appeared in 1995, in USA in 1996 and in United Kingdom in 2008 only. The varieties with this *Rz1* dominant partial resistance were symptomless but contained high virus content. However, *Rz1* is not performing equally in all genetical backgrounds or hybrid cultivars (Rush *et al.*, 2006). The total disappearance of "classic" varieties started in 1999 (KWS, 2008). After that, four different resistance genes were identified on the chromosome III (one gene with *Rz1* and Rz4 alleles and one gene with *Rz2* and Rz3 alleles) (Biancardi *et al.,* 2002; Grimmer *et al.*, 2007b). These others resistance genes were found in the *Beta vulgaris* ssp. *maritima*. The use of varieties which possessed the *Rz1* or *Rz2* gene allowed content reduction in virus until 6 X 10^4 times in comparison with the viral content in susceptible varieties (Acosta-Leal *et al.*, 2007c). Moreover, the level of resistance conditioned by the dominant *Rz2* gene appears to be higher than that conditioned by *Rz1* (Paul *et al.*, 1993b, 1993c). Nucleotide diversity was significantly higher in resistant than susceptible plants (Rush *et al.*, 2008). This suggests that nucleotide diversity is directly proportional to plant resistance strength, since the virus content was lower in *Rz2*-than in *Rz1*-plants. Also, it corroborates the higher interisolate variability observed during the breakdown of *Rz1*-mediated resistance in the field, and the high genetic stability of the wild type virus in susceptible cultivars (Acosta-Leal *et al*., 2008).The gene Rz acts in inhibiting the virus replication in the hair roots and in decreasing the virus dispersion in the taproot. Nevertheless, resistance-breaking isolates of BNYVV appeared in 2002 in France due to the systematic use of resistant varieties in infected soil in a triennial rotation. Would this have promoted faster genetic drift of the virus than that one observed in susceptible varieties? The first varieties that have shown resistance breakdown were varieties with both the *Rz1* and nematode resistance genes (ITB, personal communication, 2013). Later, it was the same with *Rz1* varieties. The sugar beet were beet-shaped "turnips" strangled, with or without root hair proliferation (Scholten and Lange, 2000; Hleibieh *et al.*, 2007). Several resistance-breaking isolates were also discovered in USA in 2002 by Liu *et al.*, 2005, Acosta-Leal *et al.* in 2007a and Pferdmenges *et al.* in 2009a. The double resistance *Rz1rz1Rz2rz2* was introduced in France in 2008 and showed better resistance against the rhizomania disease. Besides, Lennefors *et al.* (2008) shows that dsRNA-mediated transgenic resistance to *Beet necrotic yellow vein virus* in sugar beets is not affected by other soilborne and aphid-transmitted viruses. The two chalenges to control the disease seems to be the resistance against the virus and the resistance against the vector. With *Rz1* already deployed in a number of high-yielding sugar beet cultivars, the simple *Pb1*/*Pb2 (Polymyxa betae* resistant genes positioned in Chromosome VI and IX respectively*)* two-gene system represents a valuable additional target for plant breeders. A dual resistance mechanism controlled by three separate genes should confer more durable rhizomania resistance that is less susceptible to erosion by pathogen variants or high disease pressure (Asher *et al.*, 2009). Until now, seed producers have focused their selection on the virus resistance cultivars. Combined virus and vector resistance, achieved either by conventional or transgenic breeding, offers the sugar beet industry a new

approach in its continuing struggle against rhizomania (Mc Grann *et al.*, 2009).

Edaphic and phytotechnical factors

The type of soil and the irrigation influence the behavior of the vector *Polymyxa betae* and also the behavior of the sugar beet varieties. The presence or not of nematode in the rhizomania soil has also an impact on the reaction of rhizomania resistant varieties. Specific varieties has to be implemented in these fields. Some crops must be avoid in the rotation and others not before the culture of sugar beet varieties. The choice of specific varieties with specific resistances must be taken into account with a good knowledge of the characteristics of each soil. Consequently, the edaphic and phytotechnical factors are presented here. Parameters in relation with the infectious potential of the vector are also explained.

Infectious potential throughout MPN

Goffart and Maraite identified throughout the MPN method (Tuitert, 1990) in Belgium (1991) various infectious potentials comprised in a range between 0,01 to 13,6 infectious unit of vector per gram of soil (IU/g soil). The weak infectious potential of the vector (≤ 0,98 UI/g) were associated to H_20 and KCl pH ≤ 7,0 and to calcium and magnesium contents of respectively ≤ 245 and 10 mg/100 g of soil. However, the high infectious potentials of the vector correspond to pH ≥ 7,7 and to calcium and magnesium contents of respectively ≥ 365 and 20 mg/100 g. The phytotechnical practices, as liming and organic influencing the pH and calcium and magnesium contents of the soil, are in relation with the infectious potential (Hleibieh *et al.*, 2007). The spores germination and roots infection by zoospores are affected by acid pH (Abe and Tamada, 1987). They are favored by soils with neutral or basic pH, particularly when calcium and magnesium contents are respectively higher than 350 and 20 mg/100 g of soil (Goffart and Maraite, 1991).

Nematodes

In the present study, we analyzed several varieties containing the *Rz1* restistance genes in association with the nematode resistance genes. Beet cyst nematodes (BCN, *Heterodera schachtii* Schm.) form a serious pest in various parts of the beet growing area (Lange and De Bock, 1994). Partial resistance to BCN occurs in the sea beet, *B. vulgaris* subsp. *maritima* (L.) Arcang., accession BMH (Lange and De Bock, 1994). This kind of resistance appears to be controlled by a polygenic genetic system (Hijner, 1952; Heijbroek, 1977). Complete resistance to BCN was found in section *Procumbentes*, and is possibly controlled by major gene(s) (Hijner, 1952; Yu, 1984; Lange *et al.*, 1990). Using monosomic additions, one, two and three chromosomes harbouring a BCN-resistance locus were identified in *B. patellaris* Moq., *B. procumbens* Chr. Sm. and *B. webbiana* Moq., respectively (Lange *et al.*, 1990). Dominant gene(s) ($Hs1^{pat-1}$ = $Hs1^{pro-1}$) conferring full resistance to the beet cyst nematode in *B. patellaris* are

located on chromosome 1, and the other tested chromosomes of *B. patellaris* are not involved in the expression of resistance (Mesbah *et al.*, 1997). Gene(s) (*Hs1*$^{web-1}$ *& Hs2*$^{web-7}$ *& Hs3*$^{web-8}$) conferring full resistance to the beet cyst nematode in *B. webbiana* are located on chromosome 1, 7 and 8. Gene(s) (*Hs1*$^{pro-1}$ *& Hs2*$^{pro-7}$ *& Hs3*$^{pro-8}$) conferring full resistance to the beet cyst nematode in *B. procumbens* are located on chromosome 1, 7 and 8 (Heller *et al.*, 1996). The resistance-carrying chromosomes from *B. webbiana* and *B. procumbens* are homologous (Wagner *et al*., 1989; Jung *et al*., 1993). Beet cyst nematode-resistant cultivars, which were introduced recently (Cai *et al.*, 1997), originated from the homozygous inbred line B883. Tests with the resistant cultivars in climate cabinets showed a wide variety of resistance against *Heterodera schachtii* and BNYVV, expressed as average numbers of infective units per plant and percentages of resistant plants. There are differences in the degree of *H. schachtii* resistance between the cultivars, mainly caused by lower transmission rates of resistance genes (Heijbroek *et al.*, 1988; Mesbah *et al.*, 1997). Nematode varieties are classified into two categories. In the first one, the varieties called “tolerant”, which are partially resistant, assume a slight increase in nematodes. Their major interest is found in a high production potential, and a very good performance in infested soil, even slight. In the second one, the varieties “resistant”, which also possess tolerance, have for major asset a decrease of nematode populations. Their production potential is sometimes lower with a less good performance in infested and non infested soil. The germination energy of their seeds are also lower (Le Betteravier, 2010). For instance, the variety “Julietta” from KWS is a variety partial resistant to the rhizomania and tolerant to the nematodes. The variety “Correcta” is partial resistant to rhizomania and resistant to nematode. In a series of field trials at different levels of infection of *H. schachtii*, their multiplication rates on all resistant cultivars depended on the initial density, which was caused by the presence of small numbers of susceptible plants. Since tolerance to wilting was also incorporated in B883, reasonable yields were obtained in the presence of *H. schachtii*. However, at increasing initial densities of *H. schachtii*, yields decreased considerably, since penetrating juveniles cause a hypersensitivity reaction in resistant plants. Based upon the results of three series of field trials, it was concluded that resistant cultivars should preferably be applied at population densities between 500 and 2000 eggs and juveniles of *H. schachtii* per 100 ml of soil (Heijbroek *et al*., 2002). In France, resistant varieties are applied as soon as there has one cyst (ITB, personal communication, 2013). In 1999, a very high multiplication rate was detected while in 2000 the multiplication was very low, which corresponded with relatively low temperatures during spring and summer. Compared to the susceptible control, the tested cultivars showed a resistance that was most pronounced at low initial densities (Pi) of *H. schachtii*. At Pi of more than 6000 eggs and juveniles per 100 ml of soil, multiplication rates on the susceptible standard were less than 1. The results are expressed in terms of multiplication rates (Pf/Pi) and sugar yields. Cultivars with double resistance against *H. schachtii* and BNYVV behaved like those with *H. schachtii* resistance in soils infected with beet cyst nematodes, but not with BNYVV. In soils with a combined infection of *H. schachtii* and BNYVV double resistant

cultivars were far superior to single resistant ones, since damage caused by BNYVV was far more serious than damage caused by *H. schachtii*. No substantial interaction between soil pathogens nor types of resistance could be detected (Heijbroek *et al.*, 2002).

Crop management

Proper irrigation timing can maximize sugarbeet yields while minimizing disease, water costs, fertilizer leaching, and soil erosion. Crop yields can suffer from either under- or overirrigation. Underirrigation limits water flow into the plant, which reduces movement of water, nutrients, and photosynthates within the plant (Reddy *et al.*, 2007). Overirrigation reduces yield through increased incidence of disease, loss of nutrients from the soil root zone, and reduced oxygen to roots. Overirrigation can also reduce sugarbeet quality by lowering the sugar percentage. Overirrigation of sugarbeets is common, especially with furrow irrigation. Root diseases such as rhizomania and rhizoctonia root and crown rots flourish in saturated soils. If soil saturation can be minimized, development and spread of these diseases will be reduced (Reddy *et al.*, 2007).
Pesticide inputs to sugarbeet crops can often be reduced because of the lower pest pressure when these crops follow oilseed radish or white mustard (Hafez *et al.*, 2006). Compared to standard rotations, higher quality sugarbeet crops are generally grown on soils where oilseed radish or white mustard residues have been incorporated. The residues from these green manure crops add humus that improves soil tilt, water holding capacity, and nutrient availability (Hafez *et al.*, 2006). However, the culture of colza multiplies as many nematodes in the soil as the culture of sugar beet (ITB, personal communication, 2013). Consequently, the management for each field must be well thought to avoid a dramatic response.
Yields and quality are usually highest when sugarbeet follows barley or wheat in the crop rotation. Yields are usually high when sugarbeet follows corn, potatoes or summer fallow in rotation, but higher than desirable soil nitrogen levels may reduce crop quality. Three year rotations are the absolute minimum length of an acceptable rotation. Four year or longer rotations are desirable to minimize root disease, *Cercospora* leaf spot and herbicide carryover. Avoid canola in crop rotations with sugarbeet if at all possible, because it is an alternate host for sugarbeet cyst nematode. Avoid herbicide carryover that may damage sugarbeet or rotational crops. Manage fertilizer nitrogen use throughout the rotation (Hilde, 1998). Hilde, D. from American Crystal Sugar Company showed (10 year average from representative field (1987-1996)) a trend toward more sugar production per acre in rotations of five years or more.

OBJECTIVES OF THE THESIS

Objectives

The objective of the thesis is to understand the rhizomania epidemics in the Pithiviers area, through field based approaches, the spatial and temporal dynamic of BNYVV, the causal agent of rhizomania and its vector *P. betae*. The present thesis provides with an in-depth analysis of the rhizomania epidemic foci around the Pithiviers area, South of Paris, France.

Prerequisites are necessary to set up such research strategy, like a better knowledge of viruses' spatial diversity, at the sugar beet root and at the field level. Specific objectives were also tackled like the diversity of the BNYVV in a field assay implemented with three varieties, the impact of the varieties on the virus, the evolution of this diversity in a single sugar beet season, the difference in behavior between varieties with nematode resistance and without, the level of the infectious potentials in the soil and its evolution in a single sugar beet season according to the different varieties applied.

The research strategy was elaborated from the field expertise to understand the questions of a very complex disease. Consequently, limiting factors, constraints and interfering factors had to be identified: working with farmers, with different kinds of soils, environmental and climatic differences, possible interaction with other soil-borne viruses and nematodes, the length and type of crop rotation...

These constraints were balanced by a wide scale analysis combined with a long term methods, involving also high throughput methods (454 Next Generation Sequencing, NGS) and the analyses of numerous cases. In combination with disease pressure studies, the thesis has explored several tracks proposed so far to explain the emergence of resistance-breaking situation in sugar beet varieties, which contain the *Rz1* resistance gene and/or the nematode resistance gene. This has been made in the view of

providing a better understanding of the current situation and insights for an improved disease management as well as for a sustainable resistance.

This thesis was made in collaboration with the ITB which implemented varietal assays in field around the Pithiviers area. Several parameters were also measured by ITB to obtain accurate information on all samples: the nutritional factors of the plant as well as the soil composition, the presence of nematodes and the crop management (short/long rotation, irrigation, cropping history).

Thesis outline

The objective of the thesis is to understand the rhizomania epidemics in the Pithiviers area, through field based approaches.

This thesis starts with a general introduction. There is a short presentation of the rhizomania complex, the vector *Polymyxa betae*, the causal agent BNYVV, the other sugar beet soilborne viruses (pomoviruses, necroviruses, benyviruses …) and the varietal resistance in sugar beet. Moreover, there is a compilation of all the available information on the questions that were raised to explain such a problem, including nematodes, crop management (short rotation, irrigation, type of soil).

In chapter one, in collaboration with Dr Bernd Holtschulte and other researchers from KWS (Germany), a literature review on resistance to the *Beet necrotic yellow vein virus* is presented. Several concepts are discussed like the resistance-breaking, the genetic erosion, the durability of resistance, the mutant dissemination and the durability of transgenic cultivars.

The chapter two invites firstly the reader to find, in Appendix A, additional information on the methotolody used in the thesis. Methodological questions were tackled during the thesis, to unravel the gaps in the spatial dissemination of the disease both at the field and sugar beet root level. Several field assays were implemented in the Pithiviers area (South of Paris) with the contribution of the ITB. Analysis of root samples from the field and soil samples from the field were made to evaluate the rhizomania distribution in different assays. Because the viral variation questions the research strategy as well as the real significance of the current observations, the methodological questions raised to improve the reliable detection of the virus in the plant were:

- is there an intra- and inter-plot diversity of the virus and the vector ?
- what is the variability and the spatial dispersion ?
- is there an intra- and inter-beet diversity ?
- which soil samples to choose ?
- is there a viral diversity in the root samples and is there consequently a results variability ?
- is it possible to homogenize the samples ?
- how to evaluate the infection during a single sugar beet season ?
- what is the dynamic of the epidemic ?

The reliability of the results obtained direct from the field was evaluated for developing a standard methodology to assess the field assays implemented. A standard homogenization of the samples was developed to improve the current approaches. The reliability of the RT-PCR was evaluated. The repeatability was verified. Other detection technologies used were ELISA to quantify BNYVV, multiplex RT-PCR to detect different viruses, multiplex RT-mqPCR to quantify BNYVV RNA3, in situ direct hybridization and deep-sequencing (454 NGS). With regard to the soil analysis, the infectious potential of rhizomania through the technique of the Most Probable Number (MPN) were used and tested.

The chapter two is specifically dedicated to the rhizomania distribution in 2009, 2010 and 2011 in each field investigated. Firstly, this chapter presents a paper submitted in *Plant Disease* and based on the evidence for BNYVV reassortment and diversity of the p25 avirulence gene in France. This chapter aims to assess if there is a specific viral pathotype emergence as a function of the varietal sugar beet implemented in the soil and to see if there is a preferential viral pathotype development versus another.

In Chapter three, the hypothesis of a role of the genetic background of sugar beet varieties in the selection of BNYVV variants throughout micro-evolution, by using 454 NGS is discussed in a second paper that will be published in *Virus Research*, after being selected following an oral presentation at the International Plant Virus Epidemiology Conference, held in Arusha, Tanzania in January 2013. Within host evolution of BNYVV during a sugar beet crop season was evidenced.

The chapter four assesses the data obtained during four year's research through correlation studies for several diseases' indicator parameters.

General discussion, conclusion and perspectives are presented in chapter five.

CHAPTER 1

LITERATURE REVIEW

1.1 Short introduction

This literature review on the resistance of sugar beet varieties, co-reviewed with Bernd Holtschulte and other authors from KWS in Germany will give insights of some concepts as tolerance, resistance, genetic erosion, resistance-breaking strains, and resistance durability. The concepts were defined and some questions about the rhizomania problematic were investigated and compared with others plant viruses.

1.2 Literature review on resistance to the *Beet necrotic yellow vein virus*

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Abstract

The aim of this paper is on one hand to highlight the misuse of different term in different articles about virus plant which bring misunderstanding between different authors. Indeed, the terms resistance, tolerance, resistance to the pathogen, resistance to the plant, resistance-breaking, genetic erosion and durability of resistance are discussed. On the other hand, different questions

with regard to rhizomania are also discussed. The emergence of new virulent viruses could be due to a genetic erosion of resistance or a resistance break down or both. And these new virulent viruses should have a better fitness to disseminate in space and time. The occurrence frequency of such event could weaken the durability of actual resistant cultivar based on a single dominant resistance with the gene *Rz1* or transgenic genes.

Abbreviations

BBSV – *Beet black scorch virus*
BNYVV – *Beet necrotic yellow vein virus*
BSBMV – *Beet soil-borne mosaic virus*
BSBV – *Beet soil-borne virus*
BVQ – *Beet virus Q*
CP – Coat protein
MP – movement protein
PDR – pathogen-derived-resistance
RB – resistance breaking

Foreword

Though much has been published on resistance of sugar beet against rhizomania disease, there are still a lot of open and unanswered questions in such a field. The puzzle of the complex interactions between the *Beet necrotic yellow vein virus*, its vector *Polymyxa betae* and sugar beet still needs to be unravelled, and much pieces of the game are still to be found for a full understanding of this challenging problem.

1. Introduction - definitions of the concepts of resistance-breaking, genetic erosion, durability of resistance

The resistance to plant viruses has always been defined in different manners, depending on the point of view of the authors. It matters therefore to clearly express what we are speaking about when discussing such issues or using terms.

From a breeding point of view, resistance is often described with regards to the plant disease resistance standing point (no effect on yield, no symptoms, …), while the resistance to the pathogen (the virus) is mostly looked at from a plant virologist point of view (no replication, no movement, …).

It is therefore useful to distinguish firstly the tolerance in sugar beet in contrast to resistance in sugar beet and secondly the resistance to the pathogen from the resistance to the disease, though links can be assumed between both.

1.1. Tolerance in contrast to resistance

A variety called "less susceptible" is a variety on which a disease will grow less quickly than on a "susceptible" variety. We speak of partial resistance (or quantitative resistance) to describe the strength of these "less susceptible" varieties. This shows that the characterization of the level of susceptibility can be achieved by comparison with a susceptible control (Mercier, 2011). The partial resistance (Jahier *et al.*, 2009) is when the sugar beet remains infected but the multiplication rate of the pathogen is lower than in a sugar beet variety with genotype more susceptible. For example, it's usually the case for the resistance Holly or *Rz2* in sugar beet where there is lower virus content in the plant (Lennefors, 2006 and ITB 2009). The concept of partial resistance should not be confused with the concept of tolerance. The tolerance of the plant is when the multiplication rate of the pathogen (virus) is high but there are no symptoms of rhizomania clearly visible on the plant. By definition, a variety is called tolerant to a disease if, for the same amount of disease, the agronomical yield is less affected compared to a variety less tolerant (Brun, 2011). The resistance of the plant is when the multiplication rate of the pathogen (virus) in the plant is very low and there is no damage in the level of the plant. The virus is almost not encountered in the plant. For example, it's the case for transgenic plant.

1.2. Resistance to the pathogen

From a plant virologist point of view, Hull (Matthews plant virology, 2002, 4th edition) summarized different definitions & concepts previously discussed, as the fact that plants could be considered as immune (or non-host - the virus doesn't replicate in neither protoplasts nor cells, even those initially infected and no progeny virus is produced) in opposition to infectible plants (host – the virus can infect and replicate in protoplasts) – a definition proposed initially by Cooper and Jones (1983) and commonly accepted in the plant virologist community (Kang *et al.*, 2005). In 1991, Matthews defined several terms by considering the plant reaction to inoculation, with resistance being linked to the ability to produce hypersensitive reaction and to limit the spread of the virus to a limited number of cells, while susceptible plants allowed the systemic movement of the virus (Scholthof, 2005) and its replication. Susceptible plants were classified as tolerant (with little or no apparent effect on the plant, giving rise to latent infections) or susceptible (plants reacting with more or less severe plant disease).

Such definitions might be seen as particularly confusing, especially when discussing plant "resistance" issues mostly dealing with the concept of tolerance from the virology point of view, or pointing out "resistance" genes or using terms as "resistance-breaking" isolates…

1.3. Resistance to the plant disease

Host resistance to plant viruses has also sometimes being termed as specific resistance, genotypic resistance or cultivar resistance, and defined as the fact that some genotypes show heritable “resistance” to a particular viral disease whereas other genotypes are susceptible. In such resistance individuals, the virus may multiply and even move, but somewhat in a restricted manner as compared to other genotypes.

Such approaches have led to the identification of many “resistance” genes, either considered or shown as dominant (and often associated with hypersensitive reactions – avirulence) or recessive. By now, up to approximately 200 virus resistance genes have been evidenced, mostly dominant monogenic in comparison to recessive or partially dominant resistance genes.

In the literature, loss of resistance can originate from viruses (resistance-breaking virus strains) or from the plant (genetic erosion). The term “resistance-breaking” has been extensively used to pinpoint cases where a host specific resistance previously described was “broken” by variants of the virus. We have listed a number of 50 different scientific publications quoting the term in the title or the abstract, through a review of CAB abstracts from 1990 to 2010 (Lecocq & Pitrat, 1985; Lee *et al.*, 1996; Liu *et al.*, 2004; Verrier *et al.*, 2004; Rush *et al.*, 2007; Hapiak *et al.*, 2008; Poulicar *et al.*, 2010). In comparison, a Google search with the terms “Plant virus and resistance breaking” gives 210.000 hits.

In contrast, the concept of “genetic erosion” is much less used in the plant virology literature, by comparison to other field of Science. Using the same approach, we pointed only a few publications mentioning the terms. As an additional indicator, a Google search with the terms “Plant virus and genetic erosion” gives 39.000 hits.

Genetic erosion is referring to the loss of genetic diversity within a species, plant and/or viruses. The concept has been often used to explain the loss of genetic information that occurs when highly adaptable cultivars are developed and threaten the survival of their more locally adapted ancestors, which form the genetic base of the crop (Allaby, 1998). A “canonical” example of spectacular genetic erosion in plant pathology is the one of hybrid high yield maize susceptible to the blight disease caused by *Helminthosporium turcicum*, which led to the loss of up to 20% of US expected yield in 1970, because of its widespread distribution. Genetic drift can also occur in viruses. It consists in a speciation in the population with the loss of some nonuseful genes. The fitness of these genes are non adapted and the nature doesn’t keep them. The genetic erosion decreases the average fitness of the organism and the evolutionary potential of populations. Genetic drift or allelic drift is used both for virus or plant. It is the change in the frequency of a gene variant (allele) in a population due to random sampling. The alleles in the offspring are a sample of those in the

parents, and chance has a role in determining whether a given individual survives and reproduces. A population's allele frequency is the fraction of the copies of one gene that share a particular form. Genetic drift may cause gene variants to disappear completely and thereby reduce genetic variation. From a population genetics perspective, small relatively isolated populations become increasingly subject to genetic drift and inbreeding, resulting in loss of genetic variation and a decrease in fitness, a process here referred to as genetic erosion. Genetic drift will cause allele frequencies to fluctuate, which over time leads to random loss and fixation of alleles and an increase in homozygosity (Bijlsma and Loeschcke, 2012). In the majority of cases, genetic drift leads to a decrease in the genetic diversity and thus induce a genetic erosion.

"Durability of resistance" is defined as "how long a resistance can be deployed before a resistance-breaking isolates emerges". The terms durable resistance have also been used to characterize host resistance to diseases that remained effective through a relatively long period of time although resistant crop varieties are widely cultivated in an environment favourable for the disease development (Stiekema *et al.*, 1993; Swiezynski *et al.*, 1993; Turkensteen, 1993).

1.4. Sugar beet resistance to rhizomania

The pathogen causing the disease is the *Beet necrotic yellow vein virus* (BNYVV). This *Benyvirus* is a multipartite virus comprising 4 or 5 RNA single stranded, positive sense, molecules. The viral genome is described in figure 1.1, with emphasis on genes known to be involved in the interaction with the plant. These genes are the RNA2 located triple gene block, responsible for the cell-to-cell movement, the p14 gene (RNA2) and the p31 gene (RNA4), reported to act as suppressors of gene silencing (Rahim *et al.*, 2007), the p25/p26 genes (RNA3/5) described as avirulence genes. The N gene and the p31 gene with the p25/p26 genes have also been reported to be responsible for the pathogenicity of the virus.

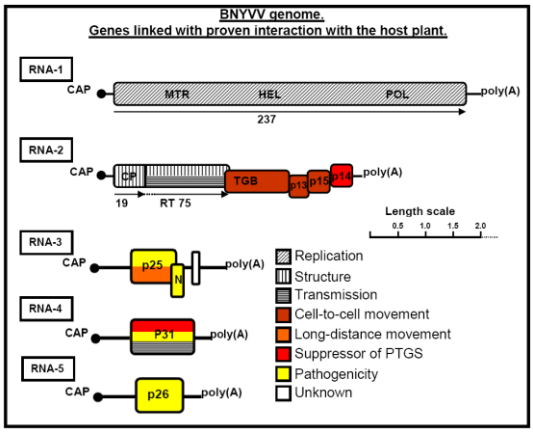

Figure 1.1. BNYVV genome

Besides the variations usually associated with RNA viruses, the BNYVV species encompass different, well-delineated, virus types. Such BNYVV pathotypes have been described based on RFLP or major sequence differences. It allows the emergence of criteria from each of the five BNYVV RNAs for the distinction of pathotype (Kruse *et al.*, 1994; Schirmer *et al.*, 2005). There are four different viral isolates, serologically similar but somewhat geographically localized:

- **A-pathotype**, comprising the four major BNYVV RNAs, is found in all European countries, in Iran, in USA, in China and in Japan.

- **B-pathotype** is particularly detected in Belgium, France and in Germany (Schmit *et al.*, 2002), but also in Switzerland, in Suede, in Poland (Borodynko et al., 2009), in China and in Japan.

- **J-pathotype** : Among the BNYVV isolates comprising a fifth RNA molecule, the J-pathotype is localized in Japan and China

- **P-pathotype** is found in France, Kazakhstan (Hleibieh *et al.*, 2007; Stacey *et al.*, 2003; Koenig and Lennefors, 2000). The P-pathotype was recently also evidenced in UK (Lemaire *et al.*, 2003, Ward *et al.*, 2007) and more recently in Iran (Merhvar *et al.*, 2009), but only comprising four RNA molecules in this last case. In recent time, this structure has more and more softened by identification of strains which are “mixtures” of the A to P-pathotypes.

This protein p25 is homodimerizable and contains an activation domain of transcription modulating the expression of cellular genes of the plant.

Much emphasis and research has been placed on p25, since the major role played in the sugar beet – virus interaction as well as significant sequences variation for p25/p26 based on non-synonymous/synonymous rate ratios (ω), indicative of selective pressure (Schirmer *et al.*, 2005). It was shown that the p25 protein is shuttling from the cell cytoplasm to the nucleus. A nuclear localisation signal as well as a nuclear export signal was evidenced, as well as a transcription activator activity in yeast one-hybrid system (Schmidlin *et al.*, 2004) (Figure 1.2). This protein is thought to enable the systemic movement of the virus in sugar beet (Lauber *et al.*, 1998b). The aa tetrad in positions 67-70 of p25 has been found to be highly variable in BNYVV sources despite the fact that the all viral genome is very stable throughout the world. Only limited internal changes in the genome have been found over a 15 years period (Koenig *et al.*, 2008; Bragard and al, 2009; Merhvar *et al.*, 2009; Meunier *et al.*, 2005; Ratti *et al.*, 2006; Schirmer *et al.*, 2005; Tamada *et al.*, 2003). It has also been suggested that mutations in the RNA3-encoded p25 protein may be related to such resistance-breaking virus variants. p25 was also identified as an avirulence factor. So far, assessment of the pathogenicity of BNYVV strains has mainly been based on symptom expression in the field (Chiba *et al.*, 2008).

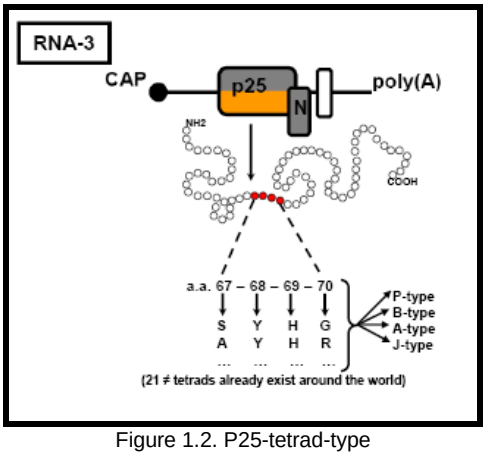

Figure 1.2. P25-tetrad-type

The type of resistance already proposed for the control of rhizomania is based on several major resistant genes known as *Rz1*, *Rz2*, *Rz3*, *Rz4*, *Rz5* (Table 1.1). Such genes are usually supported by minor resistant genes, though such are much less documented in the literature (De Biaggi, 2002, Meulemans *et al.*, 2003). In 1970s, rhizomania partial resistant sugar beets was selected in the first resistance breeding program to reduce the virus symptoms and increase the sugar yield (Fujisawa *et al.*, 1982; Bürcky, 1987; Scholten and Lange, 2000; Pferdmenges, 2007). According to Biancardi (2002), the first cultivar with “higher resistance” was Alba P (quantitative resistance).After, the variety showing higher resistance against the rhizomania was the cultivar “Rizor”. This cultivar was developed from Italian germplasm (De Biaggi, 1987). In 1983, the most important Rhizomania resistance source from the Holly Sugar Company in California, USA was the cultivar “Holly” which contained the partially dominant resistance gene named *Rz1* and others minors genes not yet identified (Lewellen *et al.*, 1987). The type of resistance of the *Rz1* beet varieties widely used through the world is considered as partially dominant resistance and in some country, at least one resistance gene is used in 70% of sugar beets (Pferdmenges, 2007). The origin of the dominant *Rz1* gene is the gene ‘Holly” from *Beta vulgaris ssp. vulgaris* in California. Several reports mentioned that under severe disease pressure, *Rz1* resistance might be inadequate (Lewellen, 1995; Paul *et al.*, 1993a; Mc Grann *et al.*, 2009). The additive effects of minor genes can explain the lower resistance stability that occurs in diploid hybrid cultivar compared to triploid (Biancardi *et al.*, 2002).

The dominant *Rz2* gene is from *Beta vulgaris ssp. maritima* in Danemark and is present in the hybrid varieties WB42 (Amiri *et al.*, 2003; Scholten *et al.*, 1999, Scholten *et al.*, 1997). The resistance level of the *Rz2* gene seems stronger than the *Rz1* gene (Hleibieh *et al.*, 2007). These two genes are present on the same chromosome III and are located in two nearby loci.

Other resistant gene like *Rz3*, *Rz4* and *Rz5* are also present on this chromosome, as well as the reported minor genes (De Temmerman *et al.*, 2009; Grimmer *et al.*, 2007a; Grimmer *et al.*, 2007b; Amiri *et al.*, 2009; Gidner *et al.*, 2005; Grimmer *et al.*, 2008; Lein *et al.*, 2007). The dominant *Rz3* gene was found recently in the hybrid variety WB41 by Gidner *et al.* (2005). There is also a probable influence of other minor genes next *Rz3* to the resistance performance. The dominant *Rz4* are from the hybrid R36 source (Grimmer *et al.*, 2008) and *Rz5* comes from the WB258 source.

However, Mc Grann *et al.* (2009) explained that the approach use for estimate the genetic distance show probably overestimation. The cartography seems not precise enough to map the major resistance genes from the different source on chromosome III. In fact, the WB41 and WB42 source had been both collected at the same time in Denmark and it seems likely that *Rz2* and *Rz3* may be the same gene, the same allelic gene. Moreover, Grimmer *et al.* (2008) mapped *Rz4* to the same location as *Rz1* and *Rz5*. This indicates that these three last genes are likely to be allelic. Lein *et al.* (2007) confirmed this by BAC clones associated with RGAs to chromosome III and found two proximal, but distant loci. The first locus was represented by alleles *Rz1*, *Rz4* and *Rz5* and the second by alleles *Rz2* and *Rz3*. Therefore, there is for the breeders a severe limitation to combine rhizomania resistance alleles from different sources reducing the five sources of resistance genes to only two sources.

In addition to the natural resistance sources found either from *Beta vulgaris* ssp. *vulgaris* or *maritima*, artificially generated resistance based on the concept of “Pathogen Derived Resistance” has also been proposed. The pathogen-derived resistance (PDR) strategy is based on the insertion of resistance genes that are derived from the pathogen (virus: MP, CP, antisens RNA, replicase) into the host plant (Baulcombe, 1996, Nelson, 1988). PDR against rhizomania has been designed based on BNYVV RNA1 replicase (Lennefors *et al.*, 2006b), RNA2 triple gene block (Lauber *et al.*, 2001) or coat protein gene (Scholten and Lange, 2000).

The control of the rhizomania disease of sugar beet is achieved mostly through the use of cultivars with monogenic *Rz1* partial resistance to the virus. Alternative sources of resistance to the vector, *P. betae,* have also been proposed as an additional strategy for controlling rhizomania. Using AFLP, the simple *Pb1/Pb2* two-gene system combined with the *Rz1* partial virus resistance is already available. QTL for resistance to both *P. betae* and BNYVV were co-localized on chromosome IV in the BC1 population (an F1 hybrid crossed with one of its parents or genetically similar individual can be termed a backcrossing1 hybrid), indicating resistance to rhizomania conditioned by vector resistance. This resistance QTL (*Pb1*) was shown in the F1 population to reduce *P. betae* levels through interaction with a second QTL (*Pb2*) found on chromosome IX (Asher *et al.*, 2009) from *Beta vulgaris ssp. maritima* in Slovenia.

Table 1. Characteristic of the different resistant genes proposed for the control of rhizomania (including resistance to the vector *P. betae*).

Hybrid	Origin	Plant gene localization	Resistance gene	Nature	Resistance level	References
Holly	*Beta vulgaris ssp. vulgaris*	Chromosome III	Rz1 (Partially dominant)	Dominant	Low-Good	Lewellen *et al.*, 1987 Scholten *et al.*, 1999 De Biaggi *et al.*, 1987
WB42	*Beta vulgaris ssp. maritima*	Chromosome III	Rz2	Dominant	Good	Lewellen *et al.*, 1987 Whitney, 1989 Amiri *et al.*, 2003
WB41	*Beta vulgaris ssp. maritima*	Chromosome III	Rz3	Dominant	Variable	Lewellen *et al.*, 1987 Whitney, 1989
R36	*Beta vulgaris*	Chromosome III	Rz4	Dominant	?	Grimmer *et al.,* 2007 Mc Grann *et al.*, 2009
WB258	*Beta vulgaris*	Chromosome III	Rz5	?	?	Grimmer *et al.,* 2007 Mc Grann *et al.*, 2009
4D6834	*Beta vulgaris*	(virus)	MP	PDR	Excellent	Lauber *et al.*, 2001
G018	*Beta vulgaris*	(virus)	Replicase	PDR/PTGS	Excellent	Lennefors *et al.*, 2006b
?	*Beta vulgaris*	(virus)	CP	PDR	Good	Scholten and Lange, 2000
?	*Beta procumbens and Beta patellaris*	Chromosome IV.1	?	Partial resistance	Excellent	Mesbah *et al.*, 1997 Paul *et al.*, 1992
		Chromosome VIII.1	?	Complete resistance	Excellent	
BB229	*Beta vulgaris ssp. maritima*	Chromosome IV	Pb1	Partial resistance	Good	Asher *et al.*, 2009
BB229	*Beta vulgaris ssp. maritima*	Chromosome IX	Pb2	Partial resistance	Good	Asher *et al.*, 2009

2 – Do we see an erosion of resistance in the rhizomania resistant cultivars in Europe/US or is it a break-down? Or both?

An erosion in the rhizomania resistant cultivars would infer a narrowing of the genes involved in the disease control. It is well known that besides the major genes that have been identified and used to introgress resistance to rhizomania, minor genes have been shown to play a role (Biancardi *et al.*, 2002 ; Meulemans *et al.*, 2003).

Is this pool of minor gene progressively diminishing within the sugar beet cultivars now in use all over the world? Do we have tools or published indications to assess such a possible diminution? Up to now, only a few papers could be linked to such hypothesis, and this is probably an explanation for the fact that genetic erosion was only seldom evoked though the role of minor genes was underlined in previous studies.

In this regard, it is worth quoting the study by Wisler *et al.* (1999) showing that differences in ELISA absorbance values (*i.e.* virus content) measured in selected cultivars closely correlated with the dosage and frequency of the Rz allele: a diploid (Rzrz) hybrid had a significantly lower absorbance value than a similar triploid (Rzrzrz) hybrid. Cultivars that segregated (Rzrz:rzrz) had higher absorbance values than uniformly resistant (Rzrz) hybrids, as was expected. Most commercial sugarbeet cultivars grown in Europe and the U.S. in 1989 were either diploid (2n =2x = 18) or triploid (2n = 3x =27) monogerm hybrids. They were produced by crossing a cytoplasmic male sterile (CMS) monogerm inbred or Fl with a diploid or tetraploid multigerm pollinator. Most cultivars developed in the United States were diploid, whereas, both diploid and triploid hybrids were being developed in Europe (Lasa *et al*., 1989). Most interesting also is the study proposed by Meulemans *et al.* (2003) where 14 female lines (11 susceptible and three resistant, homozygous for one major gene, *Beta maritima* or Rizor or Holly) were crossed with a pollinator homozygous for Holly, and checked either under bioassays or in field trials. This study stressed as Wisler *et al.* (1999) a gene dosage effect between diploid and triploid cultivars. Briefly, the resistance of the considered genes is higher when homozygous instead of heterozygous, as well as at a level of diploid instead of triploid. It also showed that combining *Beta maritima* resistance with either Rizor or Holly gave superior rhizomania control than any other combination. Interestingly, the study revealed the importance of the genetic background for the expression of the resistance, once again mentioning the importance of minor genes or so-called “resistance enhancers”. Nevertheless, the authors stressed the lack of studies with near isogenic lines to assess and compare resistance to rhizomania, which might lead to misinterpretations.

Considering these studies and thus the importance of the genetic background on the expression of resistance means it is useful considering the possibility of genetic erosion in contrast to the number of resistance-

breaking mentioned in the literature, though such mention are often quotations of initial reports…

Unfortunately, there are only poor or limited indications on the type of sugar beet varieties genetic background in relationship with the RB reported.

By now, resistance-breaking to rhizomania have been reported from only a limited number of places in the world (USA, California, Imperial valley – USA, Minnesota and a limited number of fields in several different American States – France, Pithiviers area, South of Paris – Spain, Daimiel – UK) (Table 1.2). Based on the available literature, it is somewhat difficult to appreciate the extent of the reported resistance-breaking events. Furthermore, a careful analysis denotes different situations. Clearly, the situation in fields where a fifth RNA is associated with BNYVV (like the one reported at first by Tamada *et al.*, 1989 from Japan, or the well-known situation around Pithiviers) is different from the one reported in the USA. Furthermore, even if a break-down is proven locally, it does not mean/prove that such break-down isolate will establish – Such spread will depend on virus ecology, host demography, fitness of RB isolates and especially on their ability to spread! (Rush, 2003; Maixner, 2005; Fargette *et al.*, 2006; Garret *et al.*, 2006; Chalam *et al.*, 2008; Dieryck *et al.*, 2009; Ferreira *et al.*, 2009; Cunniffe & Gilligan, 2010).

The different publications mentioning resistance-breaking for BNYVV resistance genes are listed in table 1.2. Differential behavior of sugar beet cultivars was first reported in the nineties in parallel with the distinction of different pathotypes of BNYVV by Kruse *et al.* (1994). Tamada *et al.* (1989) proposed that the variations in yield observed for *Rz1* sugar beet grown in different field conditions might be explained by differences in the viral RNA genome.

An IIRB trial was set up in 1993 to detect if the differences observed in the fields were due to the occurrence of pathotypes or should be ascribed to differences in climatic or soil conditions. The conclusions showed that the BNYVV P-pathotype, and in a lesser extend, A-pathotype, showed different levels in several sugar beet *Rz1* cultivars. The differential behaviour shown for 12 different sugar beet lines by measuring the BNYVV content in comparison with the cv. Univers raised questions or speculation about a possible selection of additional tolerance traits having been selected for several ones.

Resistance-breaking isolates where mentioned first by Liu *et al.* (2005) from the Imperial Valley, California, in the USA. Further reports were then added for several US states by Liu and Lewellen (2007) and Acosta-Leal and Rush (2007). Research focused on the diversity observed at the aa level within the p25 genes, located on BNYVV RNA3, following the publication by Tamada *et al.*, 2003, Meunier *et al.*, 2003 and Schirmer *et al.*, 2005. Several different tetrads were reported.

Ward *et al.* (2007) identified in England the P-pathotype BNYVV, which is intermediate between European and Asian RNA5. The tetrad on the p25 was identified as TYHG. Koenig *et al.* (2008) identified also a fifth RNA of J-pathotype in Germany.

Table 2. Resistance breaking events or virus variants reported for BNYVV in the literature

Location/Virus isolates	Type	Tetrad	Sugar beet cv.	Reference
J (5 RNAs)	J	Unknown	Not reported	Tamada *et al.*, 1989
Rovigo (I), Nagele (NI) Gross Gerau (G), Elst (NI) Pithiviers (F)	A B P	Unknown Unknown Unknown	Rizor, Gabriela, Stratos, Accord, FD9414, Rima Saphir, Univers, Riant, Patricia, Rialto, Elisa, H4669, S1569, Roxane, Ramona, Avantage	Heijbroek *et al.*, 1999
(J) ?	J?	Unknown	Not reported	Chiba *et al.*, 2003
USA Ca Imperial valley	A 8 isolates	Unknown	4430R, Angelina, 1927-4H5, 6600	Liu *et al.*, 2005
Worldwide study (Reassortment)	A (CP) B (CP) J (p26) P (p26)	AYHR, VLHG, ALHG, SYHG AYHR, AHHG AYHG SYHG	Roberta Roberta Roberta Roberta	Schirmer *et al.*, 2005
USA Ca, Co, Id, Mi, Ne, Or,Wa, Wy Ca	A P	ACHG, VLHG, VCHG, ALHG AFHG SYHG	4430R, G0117R, Angelina, 6600, Roberta Report of 32 RB isolates	Liu and Lewellen, 2007
(UK-MH, UK-FF) Norfolk UK	P	SYHG TYHG	None reported (Intermediate profile between asian- european isolates)	Ward *et al.*, 2007
USA Ca Imperial valley	A 56 isolates	ACHG, VLHG, VCHG, ALHG	Phenix, 4430R, 46519	Acosta-Leal and Rush, 2007
European BNYVV Saxony anhalt (G)	A B P J	ACHG, AHHG, AFHG, ALHG, AYHG, VCHG AYHR, AHHR SYHG Unknown	None reported/tested	Koenig *et al.*, 2008
Pithiviers	A,B, P Reassortment	AYHR	Koenig *et al.*, 2008	
BNYVV strains O11, GW-4 IV4, IP7, USTH, T41 Obihiro (J), Sobetsu (J), Pithiviers (F), Veneto (I), Puglia (I), Texas (USA), Tsubetsu (J), Wallerstadten (G)	A, B, J,P	Unknown	Monomodori, Rizor, Beta maritima MR0, MR1, MR2 seems to break-down Rz1 resistance ?	Chiba *et al.*, 2008 The Wallerstadten isolate
BNYVV isolates Chr – Texas (USA) Clx – Minnesota Sal – California Dwe, Mag, Spr, Tae, Nciv, Roc, Tub All from Imperial Valley, Ca, USA	A 	 ACHG ACHG ACHG VLHG/ALHG VCHG/ALHG/VRHG ALHG/ACHG ALHG	Phoenix, 4430R, Roberta, Crystal 765 (interaction rz1/WT) (interaction rz1/WT) (interaction rz1/WT) (interactionRz1/RB) (interaction Rz1/RB) (interaction Rz1/AV) (interaction Rz1/AV)	Acosta-Leal *et al.*, 2008
Rovigo (I), Groß-Gerau (G) Daimiel (S), Imperial valley (USA), Minnesota (USA)	A, B, P	Unknown	Idem as in Liu *et al.*, 2005-2007 (?)	Pferdmenges *et al.*, 2009
Rovigo (I), Groß-Gerau (G) Daimiel (S), Imperial valley (USA), Minnesota (USA)	A, B, P	Unknown	Idem as in Liu *et al.*, 2005-2007 (?)	Pferdmenges and Varrelmann, 2009
BNYVV strains S8, E12	A, B	Unknown	Partially resistant cv.	Koenig *et al.*, 2009

The idea firstly is an interaction between the p25 (a virus avirulence product-pathogenicity factor) and the plant protein from the *Rz1* gene or others proteins involved in plant defense response (a plant resistant gene) (Thiel and Varrelman, 2009). From this interaction, there is a single mutation like what happen in U. S. A. where we see a single U/C nucleotide substitution changing alanine to valine in the BNYVV p25 protein (Koenig *et al.*, 2009). This variation might be due to the selection pressure that gives an evolutionary trajectory of BNYVV with positive selection for changes required to overcome resistance. Then, there is an elimination of hitchhiking mutations through purifying selection (Acosta-Leal *et al.*, 2008). But such

selection is not sufficient for the virus to survive. After that, the mutated-virus must have the capacity to multiply itself in the host. Finally, if the fitness of the mutated–virus is sufficient, a geographical spread of the virus might occur in other plants.

To summarize, several resistance breaking events against the *Rz1* resistance to BNYVV have been reported since the nineties. Nevertheless, such event seems to rely on different viral background (presence of RNA5 or more complex interactions between BNYVV pathotypes vs single mutations (valine instead alanine on p25)). Furthermore, there are some questions regarding the fitness of the reported RB isolates and their ability to spread.

3 – Are similar examples existing from other host-virus interactions?

Plant resistance to viruses was reviewed by Fraser (1982, 1987, 1988, 1992), Khertapal *et al.* (1998), Hammond (1998b) and more recently by Goldbach *et al.* (2003), Kang *et al.* (2005) (Table 1.3), Acosta-Leal *et al.* (2005) and Carr & Loebenstein (2010). Several resistance genes have been reported to plant viruses (Posnette, 1973; Lecocq *et al.*, 2004; Stange, 2006; Ishibashi *et al.*, 2007; Larson *et al.*, 2008; Decroocq *et al.*, 2009; Gomez *et al.*, 2009; Jahier *et al.*, 2009; Matsumoto *et al.,* 2009; Moury *et al.*, 2010), mostly monogenic versus polygenic or oligogenic. Nevertheless, such pattern might simply be due to the fact that most breeding programs were aimed at searching single resistance genes.

As stated by Garcia-Arenal and McDonald (2003) and from a “plant virologist” point of view, genetic resistance often fails because a resistance-breaking (RB) pathogen genotype increases in frequency. In 1992, based on a literature analysis, Fraser published an interesting table summarizing up to 87 virus-host combinations, among which more than 75% had been overcome by virulent virus isolates. Fewer than 10% of the resistance genes listed have remained effective when tested against a wide host range of virus isolates over a long period. However, some of the virulent isolates had been found only under controlled or laboratory conditions.

Conversely also, for a few crop-virus couples, the resistance has proved to be remarkably durable. It was the case for the resistance against the *Raspberry ringspot virus* (RpRSV), *Bean common mosaic necrotic virus* (BCMNV)… Resistance is considered durable if no resistance breaking has been reported or if it has been effective for 25 years or more. By this criterion, resistance was durable in two-thirds of the analyzed pathosystems (24 out of 35). RB strains have been reported in 13 of these 24 pathosystems. In 11 pathosystems at least one resistance factor was overcome in less than 25 years, but in five of these cases there was at least one resistance factor that gave durable protection. Thus the 35 analyzed pathosystems can be split into four groups: (i) group D (11 pathosystems) in which all deployed resistance factors have been durable, and RB strains have not been reported; (ii) group DRB (13 pathosystems), in which all

deployed resistance factors have been durable, but RB strains have occurred; (iii) group ORD (five pathosystems), in which some resistance factors were overcome in less than 25 years, but others have been durable; and (iv) group O (six pathosystems), in which all deployed resistance factors have been overcome (Garcia-Arenal and McDonald, 2003).

Strikingly, on average, the life of resistance deployed against viruses is longer than that used against cellular plant pathogens (Fraile and Garcia-Arenal, 2010). A possible reason for this could be the low quantity of genes in viruses compared to e.g. fungal pathogens or bacteria or it could be the limited quantity of virulence genes in viruses adapted to the limited genetic information of the virus. Thus, the life of resistance factors deployed against fungi and oomycetes was of 7.3 years (average for 27 host–pathogen systems from data in McDonald and Linde, 2002), while for viruses it was of 12.8 years (average for 25 host–pathogen systems from data in Garcia-Arenal and McDonald, 2003).

Garcia-Arenal and McDonald (2003) analysed the evolutionary potential of 29 plant virus species involved in 35 pathosystems (Bos *et al.*, 1983; Chen *et al.*, 2002; Karadjova, 2006), and 50 resistance factors deployed against them were analyzed. Interestingly, resistance was found to be durable more often than not, in contrast with resistance to cellular plant pathogens. In a third of the analyzed pathosystems RB strains have not been reported, and in another third RB strains have been reported but have not become prevalent in the virus population (for example, the study on PVY by Acosta-Leal et al., 2005; Dale et al., 2009).

Gene and genotype flow is also an important factor in the different pathosystem for the resistance-breaking appearance. This factor quantifies the rate of migration among populations and, hence, the dispersal potential of a virus. Risk category 1 was assigned to viruses that disperse over a range of meters to kilometers (e.g., viruses that are soilborne or dispersed by contact or by vectors flying only short distances). Risk category 2 was assigned to viruses that disperse over a range of about 10 to 100 km (e.g., viruses that are persistently transmitted by flying vectors). Risk category 3 was assigned to viruses that are able to disperse hundreds or thousands of kilometers (e.g., viruses that are mainly seedborne or otherwise dispersed by humans or that persist in their long-distance-dispersing vectors) (Garcia-Arenal and McDonald, 2003).

Table 3. Viral avirulence determinants and their corresponding plant resistance genes (adapted from Kang and *et al.*, 2005)

Plant	Resistance gene	Virus	Avirulence gene	resistance Mechanism	Reported Breaking change	Resistance- References
Arabidopsis	HRT	TCV	Coat protein	HR	Not available	Oh *et al.*, 1995 Wobbe *et al.*, 1998
	RCY1	CMV	Coat protein	HR	Not available	Takahashi *et al.*, 2001
Bean	Bdm	BDMV	BV1 protein	HR	Not available	Garrido-Ramirez, 2000
Beta maritima	Rz1???	BNYVV	p25 protein	Systemic movement	Not available	Chiba *et al.*, 2008
Brassica napus	TuRBO1, TuRBO1b	TuMV	CI	HR	Single aa	Jenner *et al.*, 2000
	TuRBO3	TuMV	P3		Single aa	Jenner *et al.*, 2003
	TuRBO4,	TuMV	P3	Replication	Single aa	Jenner *et al.*, 2002
	TuRBO5		CI	HR	Single aa	
C. amaranticolor		CaMV	Gene VI product		Not available	Kiraly *et al.*, 1999 Schoelz *et al.*, 1986
Cowpea		CMV	2a polymerase	HR	Single aa	Kim *et al.*, 1997
Cucumis melo L.	nsv	MNSV	3' portion of MNSV genome	Replication	3' UTR	Diaz *et al.*, 2004
Lettuce	mol1 mol2	LMV	3' half of genome	Replication movement	Not available	Redondo *et al.*, 2001
Nicandra physaloides		PVA-M	62K and VPg	Systemic movement	Four aa	Rajamaki *et al.*, 1999
Pea	sbm-1	PSbMV	VPg	Replication	Not available	Keller *et al.*, 1998
	sbm-2	PSbMV	P3 and 6K1 cistron		Not available	Johansen *et al.*, 2001
Pepper	L1, L2, L3	TMV	Coat protein	HR	Not available	Berzal-Herranz *et al.*, 1995 de la Cruz *et al.*, 1997 Gilardi *et al.*, 1998
	pvr1 pvr2	PVY	VPg	Movement Replication	Single aa	Moury *et al.*, 2004
Potato	Nb	PVX	25 K movement protein	HR	Single aa	Malcuit *et al.*, 1999
	Nx	PVX	Coat protein	HR	Single aa	Santa Cruz *et al.*, 1993
	Rx1, Rx2	PVX	Coat protein	Replication/HR	Two aa	Bendahmane *et al.*, 1995
	Ry	PVX	Nia protease	Replication	Not known	Mestre *et al.*, 2000
Tobacco	N'	ToMV	Coat protein	HR	Single aa	Knorr *et al.*, 1988 Saito *et al.*, 1987
Tobacco	N'	TMV	Replicase (helicase domain)	HR	Several aa	Padgett *et al.*, 1997
	va	PVY	VPg	Cell-to-cell movement	Single aa	Nicolas *et al.*, 1997
N. clevelandii		TBSV	P22 movement protein	Cell-to-cell movement	Not available	Chu *et al.*, 2000 Scholthof *et al.*, 1995
Tomato	Tm-1	ToMV	Replicase	Replication	Two aa	Meshi *et al.*, 1988
	Tm-2	ToMV	30KD movement protein	HR	Two aa	Meshi *et al.*, 1989
	Tm-22					Weber *et al.*, 1993
	pot-1	PVY	VPg		Single aa	Moury *et al.*, 2004
	Sw-5	TSWV	M RNA	HR	Multiple aa	Hoffmann *et al.*, 2001
Soybean	Rsv1	SMV	HC-Pro and P3 cistron			Eggenburger *et al.*, 1997

a BYMV, Bean yellow mosaic virus; Ca MV, Cauliflower mosaic virus; CMV, Cucumber mosaic virus; LMV, Lettuce mosaic virus; MNSV, Melon necrotic spot virus; PSbMV, Pea seed borne mosaic virus; PVA-M, Potato virus A strain M; PVY, Potato virus Y; PVX, Potato virus X; TBSV, Tomato bush stunt virus; TCV, Turnip crinckle virus; TuMV,Turnip mosaic virus; ToMV, Tomato mosaic virus; TMV, Tobacco mosaic virus; TVMV, Tobacco vein mottling virus; TSWV, Tomato spotted wilt virus; SMV, Soybean mosaic virus.

4 – How can we define a break-down of resistance of Rhizomania/in virus diseases?

Taking into account the definition proposed for a resistance break-down, the fact that a resistance gene is not working anymore against a new virulent strain of the virus, resistance break-down should be, from a theoretical point of view, evaluated by comparing the effect of the virulent BNYVV isolate and/or previous one on sugar beet with the same genetic background except for the presence of the given resistance genes. One would have also to assess the level of virus within the plant for evidencing the virus ability to replicate efficiently as well as to move through the sugar beet.

For doing so, one should try to limit as much as possible interferences – the fact that for rhizomania, we are depending from an obligate endoparasite for the transmission is a constraint, since much of the trials proposed have been done using soil transmission experiments, with the problem of possible interactions/interferences with other soil-borne diseases or pathogens (nematodes, Pythium, Rhizoctonia, …). The *Polymyxa* isolates / strains must be taken into account (Legrève *et al.*, 1998, Webb *et al.*, 2000). That isolates could differ in the transmission rate of the virus and consequently BNYVV disease severity is expressed differently.

In this regard, the use of near isogenic lines would be very much useful though the access to such breeding material is not always easy for plant virologists.

The study published by R. Koenig (2009) showing that a U/C substitution within the BNYVV P25 gene promoted increased virus accumulation in roots is a very nice example of how to demonstrate the effect of a single mutation to enhance the level of BNYVV in sugar beets of the same background. Besides, Renate Koenig was able to show the effects by inoculating infectious cDNA clones without using *Polymyxa*.

In 2010, Acosta –Leal *et al.* have monitored the level of virus in the plant by an experimental approach tested with BNYVV isolates from around the world. They have made a distinction between genetic and environmental breakdown of *Rz1*-mediated sugarbeet resistance to BNYVV. The overcoming by BNYVV of the *Rz1* gene was associated with a specific mutation in the viral p25 gene (RNA3) only for isolates from the USA and Spain but not in others from around the world (Figure 1.3). Acosta-Leal suggest that BNYVV could evolve from wild type (WT) to resistance breaking (RB) variants through different mutational pathways or by environmental pathways (greater inoculum density, presence or absence of the fifth RNA of BNYVV, interaction with other viral pathogens, seed contamination, nutritionally problem, etc), thus relying on different explanations previously proposed by different authors. Rodolfo Acosta-Leal stated also that the widespread of resistant cultivars seems to favour mutations. This important factor must be also taken into account.

Nevertheless, to have the resistance of the plant no longer effective against the virus, the mutated-virus must be able to multiply within the plant and have an effect on this host plant. And at the same time, there must be dispersion and establishment of the pathogenic strain (the resistance-breaking strain) in comparison with the wild strain which are not resistant-breaking. Consequently, we have therefore the emergence of an individual RB (Duffy & Seah, 2010) and this RB strain must have the fitness required to disperse itself and settle on a large scale with the ability to overcome the resistance of the plant and to stay in a good relationship with the vector (Westwood *et al.*, 2010).

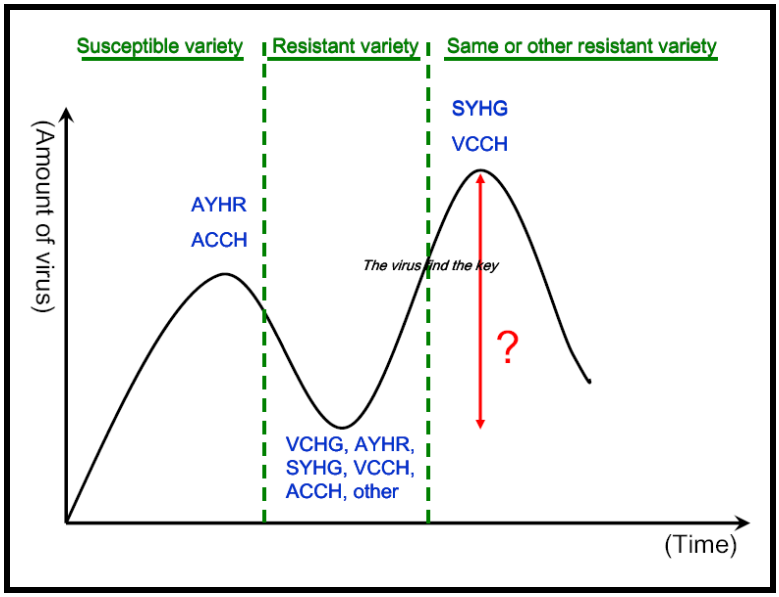

Figure 1.3. Level of different viral strains (tetrads majority and minority) as a function of different resistances of varieties of sugar beet. This is a simplified model. Of course, there are other factors that interact with the model like other hosts (wild beet and *Chenopodium*), different form of Rz resistance and environment, crop management.

Lennefors *et al.* (2008) notes in their experimentation that resistance to BNYVV was not decreased following co-infection of the plants with *Beet soil-borne virus* and *Beet virus Q*. These last two viruses share the same vector with BNYVV. Similarly, co-infection with the aphid-transmitted *Beet mild yellowing virus* (BMYV) (Stevens & Asher, 2005), *Beet yellows virus* (BYV), or with all of the aforementioned viruses were reported not to affect the resistance to BNYVV, while they accumulated in roots. These viruses are common in most of the sugar beet growing areas in Europe and world wide. However, there was a competitive interaction between BYV and BMYV in sugar beet leaves, as infection with BYV decreased the titres of BMYV (Clover *et al.*, 1999; de Koeijer *et al.*, 1999). What about interactions with *Beet soil-borne mosaic virus* (BSBMV), *Beet oak leaf virus* (BOLV) and *Beet black scorch virus* (BBSV) in Europe and USA? Are there additional hints in the literature that fungal soil borne pathogens interact with rhizomania leading to higher disease severity / damages than rhizomania alone? If we talk about rhizomania in the field are we talking about a syndrome or a disease complex? The term of syndrome has been used outside medicine to refer to a combination of phenomena, symptoms seen in association that collectively indicate or characterize a disease. A syndrome is the association of several clinically recognizable features, signs, symptoms, phenomena or characteristics essential to the diagnosis that often occur together, so that the presence of one or more features alerts to the possible presence of the others. For the most part, complex diseases are caused by a combination of genetic, environmental, and factors, most of which have not yet been identified. Considering gene-environment interactions can improve our understanding of the causes of complex disease. A complex disease is

therefore a complex trait, a polygenetic disease, a medical condition that arises from a intricate interaction of inherited–'nature' and environmental–'nature' factors.

5- How quick do the mutants ("P25" with different tetrads) disseminate (spatial and temporal dynamics of epidemics)?

It is always very difficult to predict the time a mutant will need to spread and cause an epidemic, because it's only *a posteriori* that we know for sure !

More precisely, the epidemic development of BNYVV in UK was analyzed by Stacey *et al.* (2003) through a stochastic model to predict the invasion (Gubbins *et al.*, 2000) and spread of the rhizomania (Heijbroek, 1988; Brown *et al.*, 2004; Varrelman, 2007), a highly infectious spatially heterogeneous disease. Such model could be also used to forecast the expected development of a BNYVV mutant isolate. They have also investigated the efficacy of locally and nationally reactive character of a spectrum of control strategies (novel cultivars of beet, cultivation practice, reactive containment policies,…). Larger spatial-scale processes are very successful, effective and efficient. But, it seems that a local response and a containment policy in response to the disease are largely ineffective to contain the spread (Zhang *et al.*, 2006). There is a difficulty in matching the natural scale of the epidemic, and this might hamper any control strategy.

In Europe, a P-pathotype BNYVV with a fifth RNA has been detected, more precisely around the Pithiviers region where there was beet on beet cultivation during years. This aggressive BNYVV pathotype bears on the third RNA the tetrad SYHG. The recent discovery of P-pathotype in Northern France (Pas de Calais, …) but also in UK and in Germany (Lemaire *et al.*, 2003; Schirmer *et al.*, 2005; Ward *et al.*, 2007; Koenig *et al.*, 2008; Galein *et al*, unpublished) is raising the question of the occurrence of the P-pathotype outside its original location, and thus of a possible spread of a RB isolate. Nevertheless, major differences lying within the sequences of reported isolates (Ward *et al.*, 2007; Koenig *et al.*, 2008) raise the question of occurrence through dispersion even from Asian areas than through simple spread and limited mutations from a single spot...

A BNYVV P-pathotype isolate was also reported from Kazakhstan (Koenig and Lennefors, 2000). Sequences of RNA 1, 2, 4 and 5 of BNYVV sources from Kazakhstan were almost identical to those of the P-pathotype of BNYVV which so far had been found only in a small area around Pithiviers. In parallel, a J-pathotype with also a fifth RNA was detected in Japan and China. It is now commonly accepted that the fifth RNA of the P-pathotype and the J-pathotype exacerbates the aggressiveness in symptoms induced by p25. The fifth RNA from the Pithiviers area is nevertheless considered as very different from RNA5 from various East Asian BNYVV sources (Koenig and Lennefors, 2000).

Finally, the question is “How to evaluate fitness of BNYVV pathotypes/isolates ?”. Has this isolates a good fitness to multiply itself in the plant and spread geographically and has it still a good relationship with the vector? Gilligan *et al.* (2008) have investigated an epidemiological model for invasion and persistence of pathogens motivated by questions such as “why do some diseases take off, while others die out?” They take in count a range of scales from the microscopic to the regional and gives special attention to the spatial structure of host populations and to the role of chance events in determining invasion and persistence of plant pathogens. They also take in count the long-term predictions for the persistence and the control in the managing of spread and take finally in count the notion of risk of inadvertent selection for damaging virus strains. They defined the invasion as the introduction and subsequent increase of the pathogen in a host population. Persistence is defined as an additional criterion required in which the host population provides a sufficient supply of susceptible to maintain both populations. So, there is a coexistence of the pathogen and host over long period.

Gilligan *et al.* (2007, 2008) show three simple scenarios to illustrate “no invasion”, “invasion without persistence” and “invasion with persistence” for arbitrarily scaled time and density of infected hosts. They give an example of a simple criterion for invasion states: “if, on average, one infected unit gives rise to more than one infected unit during the infectious lifetime of the donor, the pathogen will invade. If an infected unit gives rise on average to one new infection, the pathogen will persist in an endemic state, whereas below that threshold, the epidemic fades out”. They explain that an expression for invasion of the resistant type may be defined by the effectiveness of control and relative fitness of the resistant strain comparing to the sensitive.

Taking such model into account, and combining such knowledge with the fact that soil-borne viral diseases are usually expressing only years after the initial contamination, one could assume that for example BNYVV P-pathotype are able to spread, at least at field scale level as reported from field around the Pithiviers area. Nevertheless, the speed of spread is probably low. It might also be related to the type of soil as well as to crop rotation and other crop practices like irrigation. These have to be discriminated between Europe and the USA. Indeed, in USA, there is a rapid occurrence /spread of rhizomania mutants due to a different situation and mechanism for the virus evolution (Acosta-Leal and Rush, 2009). There are more “parallel mutations” like Rodolfo Acosta-Leal is mentioning.

6 – Based on the current scientific knowledge, how durable are the Rhizomania resistant cultivars (controlled by one major gene)?

Predictor model have already been proposed by Garcia-Arenal and McDonald (2003), by Janzac *et al.* (2009), by Fabre *et al.* (2009), and by

Rumbou *et al.* (2009) to predict resistance durability, based on a subset of different factors.

Garcia-Arenal and McDonald (2003) defined the durability of a resistance as an increase in the frequency of a resistance-breaking (RB) pathogen genotype able to overcome the genetic resistance of the plant. Thus the evolutionary potential of a pathogen seems a major determinant of the durability for resistance. They have tested 29 different plant virus species involved in 35 pathosystems with 50 resistance factors deployed against them. The evolutionary potential of the viruses in the 35 pathosystems was evaluated with a compound risk index based on three evolutionary factors: the population of the pathogen, the degree of recombination (Nagy, 2008; Jonson *et al.*, 2009), the type of evolution (Malcuit *et al.*, 1999, 2000) and the amount of gene and genotype flow. Their analysis indicates that evolutionary potential may be an important determinant of the durability of resistance against plant viruses or plant nematode (Bakker, 2002).

The probability of a resistance-breaking variant appearing and spreading also depends on its biological fitness in the absence of the host resistance gene, and on the type of resistance and number of resistance genes to be overcome (Harrison, 2002).

The cause of these emergences of “resistance-breakdown” is the continuous strong selective pressures on plant pathogen populations. For the moment, the durability of resistances appears after a long period of large-scale cultivation of resistant cultivars. Janzac *et al.* (2009) explain that evolutionary constraints acting on the evolution of virus avirulence factors or their diversity allow us to predict the durability of corresponding plant resistances and the pathogen emergence and re-emergence. The resistance breakdown involves modifications in the avirulence factors of pathogens and is in corresponding with the resistance plant genes in virus-plant interactions. They proposed a model predicting the potential durability of resistance genes as a function of the selective constraints applied on the corresponding aa substitutions on virus avirulence factors. They proposed an analysis of BNYVV resistance *Rz1*, for which their conclusions are that the timescale protection efficiency was from 6 to 11 years and resistance durability class 1. Their model is somewhat in disagreement with the previously proposed model of Garcia-Arenal and McDonald (2003) proposing a relatively limited evolutionary potential overall risk of 4 for the *Rz1*. In this paper, the authors mentioned grouped BNYVV together with other viruses for which resistance factors have been durable, although RB isolates are reported.

The experimental approaches don’t allow us most of the time to disentangle the interactions and the individual effects of ecological, epidemiological and evolutionary processes for the emergence of new genotypes of pathogens. Fabre *et al.* (2009) propose a model linking within- and between-host dynamics and show the key determinants of resistance durability to plant viruses. On the one hand, the different parameters taken into account are the individual plant scale, the selection processes, the drift processes, the

mutation processes and the spatial segregation of virus genotypes in their hosts. All theses parameters shape the evolution of viral populations from a set of differential equations. On the other hand, the others parameters to be involved are the host population scale and the epidemiological dynamics which shape an individual-based algorithm. Global sensitivity analyses allowed ranking the ten demo-genetic and epidemiological parameters of a model, according to their impact on the mean and variance of the risk of breakdown of a plant resistance. Demo-genetic parameters with the largest impact on the mean breakdown risk were the number and nature of mutations involved in breakdown and the fitness of mutant genotypes. However, epidemiological parameters had more influence on its standard deviation. This model might be useful to choose the potentially most durable resistance genes among a pool of candidates and allow improving durability predictions in estimating more precisely the parameters. Applied to BNYVV and *Rz1* sugar beet, the fact that a single mutation from alanine to valine, as reported by Koenig *et al.* (2009) is sufficient to overcome the resistance stress again a potential lack of durability in comparison with resistance genes requiring several mutations at the aa level to be overcome.

7 – Can we judge on the durability of a transgenic approach in Rhizomania resistance?

Predicting the durability of transgenics as far as it can be is difficult by now, due to the lack of known examples. There are only a few reported resistance-breaking with transgenic resistance to plant viruses (Sorho *et al.*, 2005; Hassani-Mehraban *et al.*, 2009). Durability of transgenic resistance will probably depend on the genetic background. Among the transgenic resistance already deployed against plant viruses (Kavanagh and Spillane, 1995; Hammond, 1998a; Lauber *et al.*, 1998a; Kiraly, 2002; Fuchs *et al.*, 2004; Sun *et al.*, 2008; Shepherd *et al.*, 2009; Pavli, 2010), one can list the papaya resistant to *Papaya ringspot virus* in Hawaï (USA) and *Plum pox virus* resistant cultivars tested at field level in Europe. Tomato resistances to *Cucumber mosaic virus* (CMV) are also used in People's Republic of China.

Several concerns have been raised regarding stability and durability of transgenic resistance to plant viruses. From a theoretical and/or practical point of view, one could imagine that large scale deployment of virus-derived transgenic plants could lead to heteroencapsidation, recombination or synergism between viruses (Bragard *et al.*, 2001; Martelli, 2001; Fuchs *et al.*, 2008; Thompson and Tepfer, 2010). Especially, the risk of plant viruses able to suppress the gene silencing mechanism therefore interfering, in the case of mixed infections, with the transgenic resistance based on such mechanism, was discussed by Ravelonandro *et al.* (2007). Besides, most of the transgenic approaches are based on PTGS or, to be more precise, on RNA interference (RNAi) (Hassani-Mehraban *et al.*, 2009). That PTGS is the natural defense mechanism of plants against viruses. And many viruses have developed also strategies to overcome (suppress) PTGS during evolution. So, in theory, one cannot exclude absolutely that a break of

transgenic resistance could happen. But, till now, there is no evidence that such has or will happen in near future which is affirmed by many studies.

The potential impact of transgenic plums and grapevines expressing viral coat protein (CP) gene constructs on the diversity and dynamics of virus populations was assessed under open and confined conditions in the frame of a research program sponsored by the European Commission (Fuchs *et al.*, 2007a, 2007b). Across all field trials conducted in different locations (France, Romania, and Spain) and environments (continental and Mediterranean), transgenic plums expressing the CP gene *of Plum pox virus* (PPV) and transgenic grapevines expressing the CP gene of *Grapevine Fanleaf virus* (GFLV) had no detectable effect on the emergence of recombinant PPV and GFLV species over eight-ten and three years, respectively. Also, no statistically significant difference was found in the number and type of aphids, including viruliferous individuals, and other arthropods that visited transgenic and nontransgenic plum trees. In addition, *Apple chlorotic leaf spot virus*, *Prune dwarf virus*, and *Prunus necrotic ringspot virus* did not influence the stability of the engineered resistance to PPV in co-infected transgenic plums over three dormancy periods. Further, under confined conditions, no recombinant virus was found to detectable level in transgenic grapevines expressing the CP gene of Grapevine virus A (GVA) or Grapevine virus B (GVB) that were challenged with the homologous or heterologous virus, despite high accumulation of transgene transcripts.

Three different transgenic sugar beet have been proposed (table 1.1), based on the BNYVV CP, TGB or replicase gene (Scholten and Lange, 2000, Lauber *et al.*, 2001, Lennefors *et al.*, 2006b). Up to now, the level of resistance reported with such transgene seems to be at least as high as the one reported for *Rz2*. If reported cases of homologous recombinations (Koenig *et al.*, 1991, Meunier *et al.*, 2003) are a matter of concern, it seems that the risk linked with the viral sequences used in the different strategies might be limited (Koenig, 2004). Similarly, the risk linked to potential heteroencapsidation was investigated without detecting any event (Koenig *et al.*, 1995). The knowledge on the interaction between BNYVV and other viruses is up to now limited, and reports are somehow contradictory. While some authors report no interactions, others like Stevens and Asher (2005) report positive interaction between BNYVV and *Beet mild yellowing virus*, interaction which could possibly hamper the transgenic strategy.

In the green-house and field trials the plants of the transgenic rhizomania resistant hybrids always contained significantly less BNYVV than plants of hybrids based on "Holly" *(Rz1).* The transgenic hybrids were also significantly more resistant than hybrids carrying resistance genes from both "Holly" and C48 *(Rz1 + Rz2 + Rz3)* when grown in the soils from Willmar, Crookston, Imperial Valley, Spain and France. The transgenic resistance was not influenced by BSBMV (Lennefors *et al.*, 2006b). In all cases, the resistant phenotype of transgenic plants carrying both transgenes was superior in comparison with the ones carrying a single transgene. Collectively, our

findings demonstrate, for a first time, that the combination of two entirely different resistance mechanisms provide high level resistance or even immunity against the virus. Such a novel approach is anticipated to prevent a rapid virus adaptation that could potentially lead to the emergence of isolates with resistance breaking properties (Pavli *et al*., 2012).

Based on what is known now, it is nevertheless difficult to predict the behaviour of the BNYVV population towards a largely deployed PDR transgene. Though one cannot rule out the possibility of resistance break down, the risk appear limited regarding to the different studies aimed at assessing specific risks. Virus-resistant transgenic plants (VRTPs) hold the promise of enormous benefit for agriculture. However, over the past ten years, questions with regard to the potential ecological impact of VRTPs have been raised. In some cases, detailed study of the mode of action of the resistance gene has made it possible to eliminate the source of potential risk, notably the possible effects of heterologous encapsidation on the transmission of viruses by their vectors. In other cases, the means of eliminating likely sources of risk have not yet been developed. When such residual risk still exists, the potential risks associated with the VRTP must be compared with those associated with nontransgenic plants so that risk assessment can fully play its role as part of an overall analysis of the advantages and disadvantages of practicable solutions to the problem solved by the VRTP (Tepfer, 2002).

8. Conslusion and recommendations

Rhizomania is a syndrome causing trouble in the interpretation of results (interaction between BNYVV and other virus diseases, soil-borne fungi, *Polymyxa* strains …). The disease of rhizomania is very complex because there are parallel mutations in different places with different types of mechanism for the emergence of RB strains. Besides, the virus BNYVV is in interaction with is vector and certainly with the *Pomoviruses* (BSBV and BVQ) associated with rhizomania. There could also have interactions with other pathogens (viruses, bacteria, fungus and nematode). Based on the literature review, the recommendations to avoid the occurrence of RB strains of BNYVV and to improve the durability of resistance gene is to take into account the genetic diversity of varieties of sugar beet when breeders select a mix of genetic to constitute the new varieties in fields in order to avoid genetic erosion with time (Palloix *et al.*, 2009). The selection of a single major gene must be outcaste and breeders should juggle with different kind of resistance (minor resistance genes, several major resistance genes against viruses, resistance genes against vector or others pathogen in interaction with the viruses, PTGS…) and avoid selection pressure with the same kind of resistance in each time. More scientific researches are needed in breeding company for these panels of resistance with new faster and improvement technologies (Bushnell *et al.*, 1998; Mouhanna, 2000; Ramamoorthy *et al.*, 2001; Smith *et al.*, 2001; Zhang *et al.*, 2002; Monti *et al.*, 2003; Lennefors, 2006a; Bondrea *et al.*, 2008) and also with regard to the evaluation of the durability of such kind of resistance. Plant breeders have to

avoid dissemination of virus diseases (Stacesmith, 1985, Boonekamp, 1989, Shpaar & Kegler, 1995) in integrating disease management for the durability of resistance against pathogens (Nakasuji, 1991; Condra, 1998; Wisler & Duffus, 2000; Varma & Mitter, 2001, Jones, 2006). The same must be done for transgenic resistance where there is only little information. Especially, for breeders, it means to be more careful before implement a new variety coming from a restrictive selection. More research should be done with regard to the interactions of the different part of the different viruses with the resistance mechanism of plants (elicitors, effectors, resistance gene,…) and the entire genome of sugar beet should be consequently better known (Bent and Mackey, 2007).

CHAPTER 2

Rhizomania reassortment and diversity

2.1 Short introduction

The chapter 2 presents a project paper. This paper demonstrates the pronouced diversity of the French *Beet necrotic yellow vein virus* (BNYVV) in the region of Pithiviers. We identified B-pathotype and P-pathotype BNYVV (p25 coding region) competition in a single sugar beet root. The control of BSBV with the *Rz1rz1Rz2rz2* varieties was evidenced. We observed also a decrease of the infectious potential with *Rz1rz1* varieties with nematode resistance. In case of mixed BNYVV pathotypes infections, these varieties showed even lower root severity than the other varieties.
Complement information for the materials and the methodologies used in the thesis are detailed in appendix A.

2.2 Paper submitted in the journal "*Plant Disease*"

Evidence for *Beet necrotic yellow vein virus* Reassortment and Diversity of the p25 Avirulence Gene in France.

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Abstract

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Since 1990 in the Pithiviers region of France, the value of producing sugar beet, *Beta vulgaris* L., has changed significantly and the causal agent of rhizomania, *Beet necrotic yellow vein virus* (BNYVV), has endured strong selection pressure. Field research was conducted to investigate both the virus (RNA2, 3 & 5) and the vector, *Polymyxa betae* as well as other Pomoviruses within cultivars fo find a hypothetical basis for changes in infectivity of BNYVV. The situations in the USA and France were shown to be completely different. BNYVV was detected in 835 of the 1,058 samples collected between 2008 and 2012. Twenty-one variants of the highly variable aa tetrad at positions 67–70 of p25 were identified. There was considerable diversity in the RNA3 tetrads, which is a resistance-breaking indicator. The first variant, AYHR, was found most commonly followed by SYHG. These tetrads in mixed infection exhibited competition within a root. There was evidence of BNYVV reassortment in 20% of the samples. Moreover, the cultivars with *Rz1rz1Rz2rz2* genotypes had fewer root symptoms and lower virus infectious potential at the end of the growing season, and had lower titer with *Beet soil-borne virus* (BSBV), a *Pomovirus* often found in association with rhizomania.

Rhizomania is one of the most challenging diseases of sugar beet because of its complexity and the difficulty in maintaining plant resistance source. Since its discovery in 1952 (Canova), rhizomania is now widespread

in most sugar beet growing countries (Koenig *et al.*, 2008; Chiba *et al.*, 2011). The disease is caused by *Beet necrotic yellow vein virus* (BNYVV) (Tamada and Baba, 1973), a soil-borne virus transmitted by the plasmodiophorid *Polymyxa betae* (Keskin, 1964). Other soil-borne viruses also have the same vector and are frequently found in association with rhizomania: *Beet soil-borne virus* (BSBV) (Meunier *et al.*, 2003), *Beet virus Q* (BVQ) (Crutzen *et al.*, 2009) and *Beet soil borne mosaic virus* (BSBMV) (Mahmood and Rush, 1999).

The BNYVV genome comprises of four or five positive sense single stranded RNAs. BNYVV RNA1 and RNA2 molecules are needed for infection (Bouzoubaa *et al.*, 1986), but additional RNAs enhance pathogenicity and general fitness. The *p25* gene on RNA3 was reported to act as an avirulence gene (Pferdmenges, F. 2007; Chiba *et al.*, 2011). The *p31* gene on RNA4 is involved in both transmission by the vector *Polymyxa betae*, pathogenicity and suppressing post-trancriptional gene silencing (Guilley *et al.*, 2009). The virus is also sometimes associated with an additional RNA, RNA5, which is known to enhance virulence of symptoms in sugar beet (Tamada *et al.*, 1996). This RNA codes for the protein p26 and contains also a transcriptional activation domain (Covelli *et al.*, 2009). In terms of evolution, it is thought that BNYVV originated from Asia, and that its RNA5, initially present, has been lost over time (Chiba *et al*., 2011).

Rhizomania is primarily managed through the use of the *Rz1* gene (Holly) which allows for partial resistance. This source of resistance was isolated from *Beta vulgaris* in California, but the *Rz1* gene has been incorporated broadly into sugar beet and is used throughout most production regions, worldwide. The tolerance of sugar beet cultivars is linked to a restriction of virus multiplication and translocation in taproots rather than in rootlets (Scholten *et al.*, 1994; Tamada *et al.*, 1999; Chiba *et al.*, 2008). In intensively cropped areas, resistance began to breakdown in cultivars with *Rz1* (with nematode resistance first) and then *Rz1* + *Rz2* due to the emergence of resistance-breaking (RB) viral strains in both North America and Europe (Liu *et al.*, 2005; Pferdmenges and Varrelmann, 2009). The emergence of *Rz1* resistant-breaking isolates has now been reported in sugar beet growing areas in Asia (Chiba *et al.*, 2011), Europe (Bornemann and Varrelmann, 2013), and in the USA (Acosta-Leal and Rush, 2007), from most US production regions (Liu and Lewellen, 2007), but only in California and Minnesota. Although the diversity of the BNYVV genome is considered as limited in comparison with other plant viruses, a set of four consecutive amino acids, or “tetrad”, ensures a strong positive selection pressure on the *p25* gene (Meunier *et al.*, 2003; Schirmer *et al.*, 2005). This tetrad was linked with resistance-breaking (RB) events, i.e. with emergence of symptoms in cultivars considered tolerant or resistant (Acosta-Leal, 2007 and 2010; Acosta-Leal and Rush, 2007; Koenig *et al.*, 2008 and 2009a; Liu and Lewellen, 2007).

Since 1994, based initially on RFLP studies, three pathotypes named A, B and P have been described within the BNYVV species and used to differentiate isolates (Kruse and Koenig, 1994; Koenig *et al*., 1997). Later on, an additional J-pathotype was proposed for Asian strains with an additional RNA5 (Schirmer *et al.*, 2005). Based on an extensive comparison of 73

isolates of worldwide origin, Chiba *et al.* (2011) proposed phylogenetic evolutionary pathways for BNYVV populations, with up to eight different groups with different geographical distributions.

The presence of a fifth RNA molecule, associated with the viral genome, was reported from several places in Europe, nothern France, UK and Germany. The P-pathotype was found in Pithiviers in France (Koenig *et al*., 1997; Heijbroek *et al.*, 1999), Kazakhstan (Koenig and Lennefors, 2000) and UK (Harju *et al*., 2002; Ward *et al*., 2007) while the J-pathotype was recorded in China and Japan (Tamada *et al*., 1989; Kiguchi *et al*., 1996; Miyanishi *et al*., 1999). A RNA5-containing BNYVV genotype closely resembling the Chinese isolate Har4 was also identified in Germany (Koenig *et al*., 2008). In general, no RNA5 has been associated to the A-pathotype or the B-pathotype (Peltier *et al.*, 2008). In Iran, the tetrad SYHG associated to the P-pathotype has been found without a fifth RNA in fields with severe symptoms (Mehrvar and Bragard, 2009b).

Despite the relative diversity of BNYVV strains, mixed infections have only been rarely reported (Koenig *et al.*, 2009a). Ratti *et al.* (2006) detected only a single A/B pathotype infection out of 72 European samples. Similarly, evidences for reassortements have been reported from UK (Ward *et al.*, 2007), France (Koenig *et al.*, 2009a) from localized areas or Asia (Chiba *et al*., 2011, Li *et al*., 2008).

Here we report rhizomania surveys conducted in the Pithiviers area from 2008 to 2011, complementary to the study of Schirmer *et al.* (2005), based on extensive field sampling and testing, combined with field assays set up to follow rhizomania development over the sugar beet growing season. Different indicators linked to the disease were used such as the infectious potential through the Most Probable Number (MPN) of the vector and BNYVV in the soil, the presence of BNYVV, BVQ and BSBV detected by reverse transcriptase polymerase chain reaction (RT-PCR), the BNYVV p25 aa tetrad and finally the contents of the virus in each sample by enzyme linked immunosorbent assay (ELISA) and reverse transcriptase multiplex quantitative real-time polymerase chain reaction (RT-mqPCR). The impact of the disease has been also measured in field to evaluate symptoms through the root severity of the sugar beet roots in the fields.

Materials and Methods

The present study concerns the BNYVV diversity analysis in field assays between 2008 and 2011. The data recorded for each assay included: management of crop residues, tillage, type of irrigation, soil pH, soil type, weather, root severity, the presence or absence of rhizomania beets earlier, planting date, and the presence of cyst nematodes (cyst and larvae of *Heterodera Schachtii*). In a 150 km^2 area corresponding to locations with severe rhizomania foci, targeted BNYVV genes in samples were analyzed by RT-PCR and sequencing.

Field assays and plant materials. Ten field assays were conducted each year from 2009 to 2011. Two French regions were mainly studied, ‘Loiret’ and ‘Seine-et-Marne’, but other regions were also investigated such

as ‘Aisne’, ‘Eure-et-Loire’, ‘Aube’, ‘Oise’, ‘Yvelines’ and ‘Essonne’ (Figure 2.1).

Between 2009 and 2011, thirteen cultivars with different resistance types were used in field assays. The cultivars were coded and presented in Table 2.1. The fields assays were also coded (L for ‘Loiret’, I for ‘Seine-et-Marne’). Each field was different from the others except for the field coded EL09 and EL11 in Dambron which are the same. For each assay, the field was subdivided in three varietal bands and each band was subdivided in four zones. There were consequently 12 zones per field. The fields which contained cyst nematodes were planted with resistant cultivars. In 2009 and 2011, there were mixed samples (one mixed sample was represented by five to six different roots) per zone pooled for the analysis. In 2010, there was only a single root per mixed sample analyzed to observe the diversity in a single root. Table 2.2 also shows the cultivars in the diseased fields (between 2008 and 2011) that were analyzed in patches of diseased plant with symptoms. A total of 1,058 samples were analysed over three years.

Assessment of infectious potential in soils by bioassays in controlled conditions. To evaluate the infectious potential of a soil (vector effectively germinated amongst all the resting spores in the soil), bioassays have been set up in controlled conditions in a greenhouse. Two samples of soils per year were made in each zone of each field assay, one just before the seedling and the other one at the harvesting period. Therefore, there were 2 X 12 samples per site and per year. The estimation was based on the theory of “Most Probable Number” (MPN) of the vector and the virus in the soil (Tuitert, 1990 and 1994; Goffart and Maraite, 1991). Here, the dilution range of a soil sample was 5^{-1} to 5^{-7} and the susceptible (r*z1rz1rz2rz2*) variety (Cadyx) was used (Blunt *et al.*, 1991). The plants were analyzed for the presence of *P. betae* and BNYVV (RNA3) after an incubation period of six weeks to obtain the virus MPN and the vector MPN (infective units per gram of soil). These infectious potentials were calculated through the MPN calculator developed by Parnow in 1972. Finally, the MPN obtained in September represents the inoculum in the beginning of the culture less the germinated inoculum plus the intake of new inoculum by the plant.

Total RNA extraction. Each rootlet was lyophilized (Lyophilisator Heto PowerDry PL6000-55 Thermo by Thermo Scientific, France) and then homogeneized. The total RNA was extracted from 100 mg of homogenized root powder from each sample using the RNeasy extraction kit (Qiagen, Hilden, Germany) or similar technique.

RT-PCR detection of the viruses and vector. A duplex RT-PCR assay was used for the detection of RNA3 and RNA5 of BNYVV. For the RNA3, the primer pair 5’-CAGTTTATGATTTAGGGCACA-3’ / 5’-ATCATCATCAACACCGTCAG-3’ was used to amplify the *p25* gene by RT-PCR. For the RNA5, the primer pair 5’-ATGTTTGTTGGTCCCCCGCT-3’ / 5’-CGAGCCCGTAAACACCGCAT-3’ was used to amplify a portion of the *p26* gene. To analyze the bio-assay, a duplex RT-PCR assay was used for the

detection of RNA3 in the BNYVV and ITS1 in the *P. betae* with primers according to Legrève *et al.* (2003). The presence of other viruses (BVQ and BSBV) was evaluated through a multiplex RT-PCR assay (Meunier *et al.*, 2003). *Beet soilborne mosaic virus* (BSBMV) another virus transmitted to sugar beet by *P. betae* was tested with primers developed by Mahmood and Rush (1999). For detection of *Beet black scorch virus* (BBSV), transmitted by *Olpidium brassicae*, primers designed by Weiland *et al.* (2007) were used.

Sequencing. Before sequencing, the PCR products were purified by the MSB® Spin PCRapace Kit (Invitek GmbH, Berlin, Germany). The nucleotide sequences of the PCR products from *p25* were obtained using an ABI377 Sequencer-Genetic Analyser and the "Big Dye Terminator Cycle Sequencing Kit" (Applied Biosystems). The identity of the sequences were analyzed by Blastn in NCBI. The Clustal W program of the Genetic Computer Group (Devereux *et al.*, 1984) was used to align the sequences. The Translate tool of the Expasy Proteomics Server tools was used to translate the nucleic acid sequence into amino acid (aa) sequence. The Invitrogen Vector NTI Advance 10 program was used to combine two reads (forward and reverse) per gene in a consensus sequence.

Quantification of BNYVV. In order to quantify the B-pathotype (tetrad AYHR) and the P-pathotype RNA3 (tetrad SYHG) of BNYVV, a RT-mqPCR was used. The forward (5'-TTCACTGGTGTATCACAATAAIACTAAACG-3') and reverse (5'-GAGGTAIAGAACATAACATTACTCTACATAAGG-3') primers were designed using Beacon Designer 7.91 (Premier Biosoft International) to amplify the region around the highly variable tetrad. For the multiplex quantification (Bertolini *et al.*, 2001), the Taqman® probes AYHR-probe, (5'-TTTCGTGGTTTATTGTGTGCTTATCATAGGC-3') and SYHG-probe (5'-TTTCGTGGATTATTGTGTTCTTATCATGGGC-3') were developed. The probe and primers were provided by Eurogentec S.A. (Liège, Belgium). The real-time quantitative PCR was performed using the iCycler IQ™ or the CFX96™ Real-Time System C1000 Touch™ Thermal Cycler of Biorad (Hercules, CA). The acetolactate synthase (ALS) endogenous control gene was used as an intern positive control (IPC) normalized, to limit bias in the real-time RT-mqPCR (Huggett *et al.*, 2005). The ALS primers were the following: forward (5'-CTACCAACCTCGTATCAG-3') and reverse (5'-GGCTTCCTTAACAATTCTAG-3'). The taqman ALS-probe Cy5-5'-TCTTGCTGACGCTCTCCTTGAT-3'-BHQ2 was used. Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) using the DSMZ kit (Leibniz Institute, Germany) was also carried out using polyclonal antibodies raised against BNYVV as described by Koenig *et al*. (1997).

Table 2.1. Locations were field assays were conducted in France.

Field assays

Year	Region	Field code	Variety	Variety code	Resistance type	Seed company
2009	Boynes 1	AL09	Ludwina	V1	*Rz1rz1 & Rz2rz2*	KWS
			Sophia	V3	*Rz1rz1*	KWS
			Python	V5	*Rz1rz1*	SES Vanderhave
	Boynes 2	BL09	Ludwina	V1	*Rz1rz1 & Rz2rz2*	KWS
			Python	V5	*Rz1rz1*	SES Vanderhave
			Sophia	V3	*Rz1rz1*	KWS
	Teillay-le-Gaudin 1	CL09	Béring	V8	*Rz1rz1 & Rnematode*	Strübe
			Annouschka	V9	*Rz1rz1 & Rnematode*	KWS
			Julietta	V4	*Rz1rz1 & Rnematode*	KWS
	Sougy 1	DL09	Julietta	V4	*Rz1rz1 & Rnematode*	KWS
			Annouschka	V9	*Rz1rz1 & Rnematode*	KWS
			Béring	V8	*Rz1rz1 & Rnematode*	Strübe
	Dambron 1	EL09	Julietta	V4	*Rz1rz1 & Rnematode*	KWS
			Béring	V8	*Rz1rz1 & Rnematode*	Strübe
			Fiorenza	V13	*Rz1rz1 & Rnematode*	KWS
	Poupry 1	FL09	Annouschka	V9	*Rz1rz1 & Rnematode*	KWS
			Julietta	V4	*Rz1rz1 & Rnematode*	KWS
			Béring	V8	*Rz1rz1 & Rnematode*	Strübe
	Mondreville 1	AI09	Ludwina	V1	*Rz1rz1 & Rz2rz2*	KWS
			Sophia	V3	*Rz1rz1*	KWS
			Python	V5	*Rz1rz1*	SES Vanderhave
	Chenou 1	BI09	Python	V5	*Rz1rz1*	SES Vanderhave
			Ludwina	V1	*Rz1rz1 & Rz2rz2*	KWS
			Sophia	V3	*Rz1rz1*	KWS
2010	Boynes 3	AL10	Ludwina	V1	*Rz1rz1 & Rz2rz2*	KWS
			Python	V5	*Rz1rz1*	SES Vanderhave
			Sophia	V3	*Rz1rz1*	KWS
	Yèvres-la-Ville 1	BL10	Sophia	V3	*Rz1rz1*	KWS
			Ludwina	V1	*Rz1rz1 & Rz2rz2*	KWS
			Python	V5	*Rz1rz1*	SES Vanderhave
	Teillay-le-Gaudin 2	CL10	Julietta	V4	*Rz1rz1 & Rnematode*	KWS
			Béring	V8	*Rz1rz1 & Rnematode*	Strübe
			Bison	V10	*Rz1rz1 & Rnematode*	SES Vanderhave
	Sougy 2	DL10	Bison	V10	*Rz1rz1 & Rnematode*	SES Vanderhave
			Julietta	V4	*Rz1rz1 & Rnematode*	KWS
			Béring	V8	*Rz1rz1 & Rnematode*	Strübe
	Dambron 2	EL10	Adriana	V12	*Rz1rz1 & Rnematode*	KWS
			Julietta	V4	*Rz1rz1 & Rnematode*	KWS
			Béring	V8	*Rz1rz1 & Rnematode*	Strübe
	Poupry 2	FL10	Julietta	V4	*Rz1rz1 & Rnematode*	KWS
			Béring	V8	*Rz1rz1 & Rnematode*	Strübe
			Bison	V10	*Rz1rz1 & Rnematode*	SES Vanderhave
	Corbeilles-en-Gatinais	GL10	Sophia	V3	*Rz1rz1*	KWS
			Python	V5	*Rz1rz1*	SES Vanderhave
			Ludwina	V1	*Rz1rz1 & Rz2rz2*	KWS
	Mondreville 2	AI10	Ludwina	V1	*Rz1rz1 & Rz2rz2*	KWS
			Sophia	V3	*Rz1rz1*	KWS
			Python	V5	*Rz1rz1*	SES Vanderhave
	Chenou 2	BI10	Sophia	V3	*Rz1rz1*	KWS
			Ludwina	V1	*Rz1rz1 & Rz2rz2*	KWS
			Python	V5	*Rz1rz1*	SES Vanderhave
	Gironville 1	CI10	Python	V5	*Rz1rz1*	SES Vanderhave
			Sophia	V3	*Rz1rz1*	KWS
			Ludwina	V1	*Rz1rz1 & Rz2rz2*	KWS
2011	Boynes 4	AL11	Britta	V2	*Rz1rz1 & Rz2rz2*	KWS
			Python	V5	*Rz1rz1*	SES Vanderhave
			Sophia	V3	*Rz1rz1*	KWS
	Yèvres-le-Chatel 1	BL11	Rosalinda	V7	*Rz1rz1*	KWS
			Magellan	V6	*Rz1rz1*	SES Vanderhave
			Britta	V2	*Rz1rz1 & Rz2rz2*	KWS
	Teillay-le-Gaudin 3	CL11	Baobab	V11	*Rz1rz1 & Rnematode*	SES Vanderhave
			Julietta	V4	*Rz1rz1 & Rnematode*	KWS
			Bison	V10	*Rz1rz1 & Rnematode*	SES Vanderhave
	Baigneaux	DL11	Baobab	V11	*Rz1rz1 & Rnematode*	SES Vanderhave
			Julietta	V4	*Rz1rz1 & Rnematode*	KWS
			Bison	V10	*Rz1rz1 & Rnematode*	SES Vanderhave
	Dambron 1	EL11	Julietta	V4	*Rz1rz1 & Rnematode*	KWS
			Baobab	V11	*Rz1rz1 & Rnematode*	SES Vanderhave
			Bison	V10	*Rz1rz1 & Rnematode*	SES Vanderhave
	Poupry 3	FL11	Baobab	V11	*Rz1rz1 & Rnematode*	SES Vanderhave
			Julietta	V4	*Rz1rz1 & Rnematode*	KWS
			Bison	V10	*Rz1rz1 & Rnematode*	SES Vanderhave
	Yèvres-le-Chatel 2	GL11	Sophia	V3	*Rz1rz1*	KWS
			Python	V5	*Rz1rz1*	SES Vanderhave
			Britta	V2	*Rz1rz1 & Rz2rz2*	KWS
	Mondreville 3	AI11	Python	V5	*Rz1rz1*	SES Vanderhave
			Sophia	V3	*Rz1rz1*	KWS
			Britta	V2	*Rz1rz1 & Rz2rz2*	KWS
	Moisancelle-en-Gatinais	BI11	Python	V5	*Rz1rz1*	SES Vanderhave
			Sophia	V3	*Rz1rz1*	KWS
			Britta	V2	*Rz1rz1 & Rz2rz2*	KWS
	Gironville 2	CI11	Python	V5	*Rz1rz1*	SES Vanderhave
			Britta	V2	*Rz1rz1 & Rz2rz2*	KWS
			Sophia	V3	*Rz1rz1*	KWS

Table 2.2. Plant material from farmer's fields.

Farmer's fields (patches)

Year	Region	Variety	Variety code	Resistance type	Seed company
2008	Mérouville & Sougy & Ruan & Bricy & Baigneaux & Trinay & Bucy-le-Roi & Bondaroy	Julietta	V4	*Rz1rz1 & Rnematode*	KWS
	Chézy-sur-Marne	Python	V5	*Rz1rz1*	SES Vanderhave
	Dadonville	Sporta	V17	*Rz1rz1*	Syngenta
	Boutigny-sur-Essonnes & Saint-Pierre	Ardan	V18	*Rz1rz1*	Florimond Desprez
	Chézy-sur-Marne & Aufferville	Jetta	V19	*Rz1rz1*	Ringot Betteraves
	Mérouville & Bondaroy	Zanzibar	V20	*Rz1rz1*	SES Vanderhave
	Ramoulu	Galactica	V21	*Rz1rz1*	KWS
	Thiersanville	Nordika	V22	*Rz1rz1*	KWS
	Bondaroy	Cheyenne	V23	*Rz1rz1*	SES Vanderhave
	Bondaroy	Danube	V24	*Rz1rz1*	Florimond Desprez
	Sougy	Narcos	V25	*Rz1rz1*	Florimond Desprez
	Bondaroy	Carissima	V26	*Rz1rz1*	Betaseed
	Bondaroy	Rigel	V27	*Rz1rz1*	Betaseed
	Bondaroy	Zoulou	V28	*Rz1rz1*	Ringot Maribo
	Bondaroy	Emilia	V29	*Rz1rz1*	KWS
	Bondaroy	Othello	V30	*Rz1rz1*	Ringo Maribo
	Bondaroy	Sculta	V31	*Rz1rz1*	Hilleshog NK
	Bucy-le-Roi & Autroche & Saint-Pierre	Annalisa	V32	*Rz1rz1 & Rnematode*	KWS
	Bondaroy	Encarta	V33	*Susceptible*	Syngenta
	Bondaroy	Harmonia	V35	*Rz1rz1*	Betaseed
2009	Yèvres-la-Ville & Bondaroy	Ludwina	V1	*Rz1rz1 & Rz2rz2*	KWS
	Yèvres-la-Ville & Bondaroy	Britta	V2	*Rz1rz1 & Rz2rz2*	KWS
	Montargis & Yèvre-la-Ville & Bondaroy	Python	V5	*Rz1rz1*	SES Vanderhave
	Yèvres-la-Ville & Bondaroy	Magellan	V6	*Rz1rz1*	SES Vanderhave
	Gidy & Ruan	Béring	V8	*Rz1rz1 & Rnematode*	Strübe
	Yèvres-la-Ville & Bondaroy	Cétus	V16	*Rz1rz1*	Deleplanque
	Yèvres-la-Ville & Bondaroy	Encarta	V33	*Susceptible*	Syngenta
	Yèvres-la-Ville & Bondaroy	Magistral	V34	*Rz1rz1*	SES Vanderhave
	Yèvres-la-Ville & Bondaroy	Harmonia	V35	*Rz1rz1*	Betaseed
	Yèvres-la-Ville & Bondaroy	Débora	V36	*Rz1rz1*	KWS
	Yèvres-la-Ville & Bondaroy	Antoinetta	V37	*Rz1rz1*	KWS
2010	Chezy	Ludwina	V1	*Rz1rz1 & Rz2rz2*	KWS
	Sougy	Julietta	V4	*Rz1rz1 & Rnematode*	KWS
	Neuville-au-bois	Magellan	V6	*Rz1rz1*	SES Vanderhave
	Sougy	Bison	V10	*Rz1rz1 & Rnematode*	SES Vanderhave
	Mayvillers	Adriana	V12	*Rz1rz1 & Rnematode*	KWS
	Poupry	Massima	V14	*Rz1rz1 & Rnematode*	Betaseed
	Laon	Unknown	V15	*Rz1rz1*	Unknown
	Baigneaux	Cétus	V16	*Rz1rz1*	Deleplanque
	Chezy	Zoulou	V28	*Rz1rz1*	Syngenta
	Chezy	Othello	V30	*Rz1rz1*	Ringot Maribo
	Pierrefonds	Encarta	V33	*Susceptible*	Syngenta
	Voué	Unknown	V39	*Rz1rz1*	Unknown
2011	Mérouville	Britta	V2	*Rz1rz1 & Rz2rz2*	KWS
	Teillay-le-Gaudin	Julietta	V4	*Rz1rz1 & Rnematode*	KWS
	Sougy	Magellan	V6	*Rz1rz1*	SES Vanderhave
	Janville	Rosalinda	V7	*Rz1rz1*	KWS
	Sougy	Belino	V38	*Rz1rz1*	Florimond Desprez

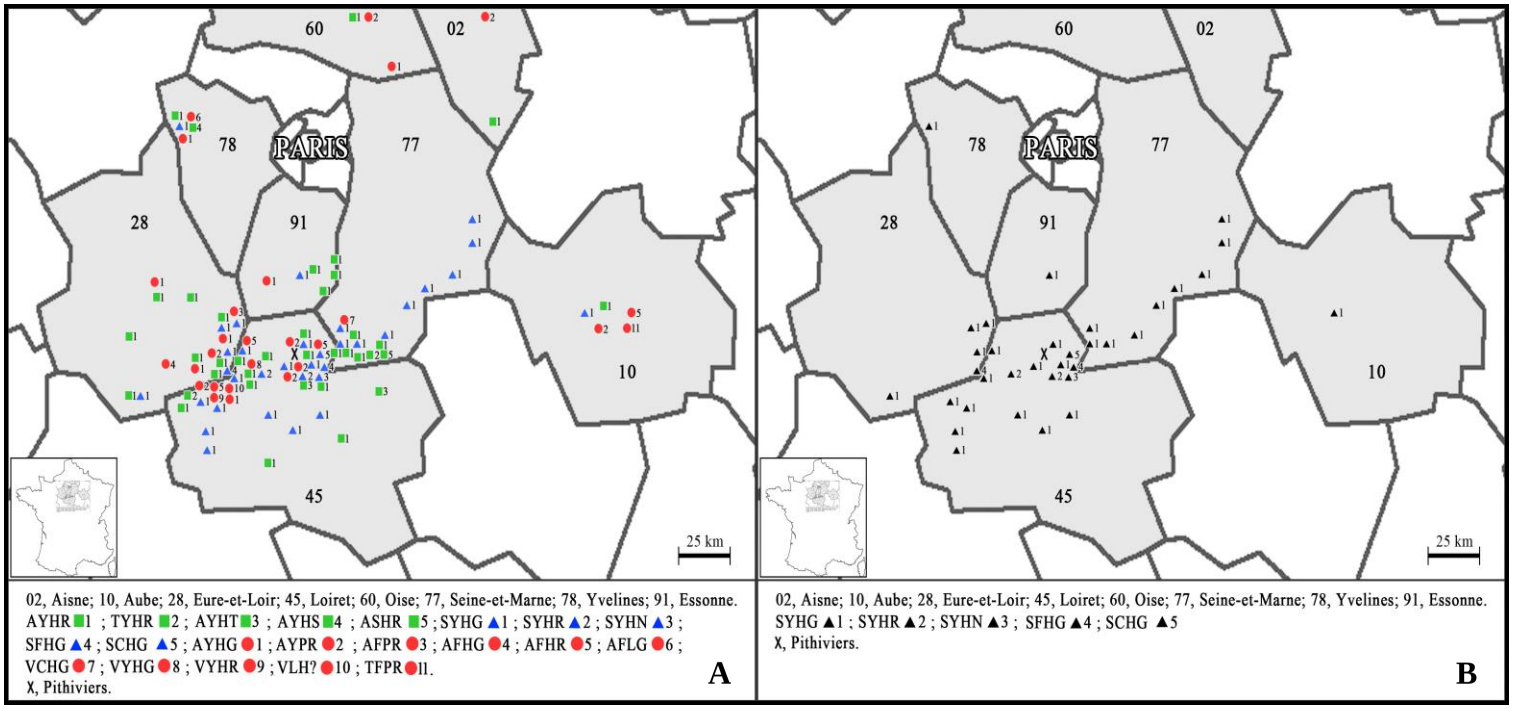

Fig. 2.1. Distribution of *Beet necrotic yellow vein virus* (BNYVV) and the associated tetrads in the Pithiviers region in France. Panel A shows all the tetrad diversity of BNYVV and Panel B shows the P-pathotype associated tetrad dispersion in the Pithiviers area.

Results

Diversity of the BNYVV p25 tetrads in the French Pithiviers area. The pathotype of p25 aa tetrads detected between 2008 and 2012, and their location in the Pithiviers area were determined (Table 2.3 and Fig. 2.1 respectively). The total of 1,058 samples were evaluated and 835 were positive for BNYVV. Amongst these samples, 482 isolates were found evaluated from individual sugar beet roots (Table 2.3) and 353 isolates were evaluated from mixed sugar beet roots.

Amongst the 835 BNYVV-infected isolates, only a single mutation alanine-valine in the tetrads (0.65% of the total isolates) was detected. In individual sugar beet root, single infection was observed in 80% of the samples and the other 20% were mixed infection (Table 2.3). In this study, 56% of the positive samples had the B-pathotype AYHR tetrad (frequency occurrence based on 835 positively infected samples) and 32% had the P-pathotype SYHG tetrad. The A-pathotype tetrads AHHG and ALHG were not found in the roots samples. Conversely, tetrads (SYHG, AYHR, AYPR, AFHG, AFHR, VCHG, and VLHG) linked with resistance-breaking events were found (Table 2.3). The sugar beet variety where the highest tetrad diversity contained both the *Rz2* and *Rz1* resistant genes. In mixed sugar beet roots, other tetrads were also found: 0.13% for B-pathotype AYHS and ASHR, 0.13% for P-pathotype SYHN, SFHG and SCHG, and 0.13% for A-pathotype AFLG and VYHG.

Large presence of BNYVV "reassortant" strains. Table 2.3 shows also 26% of single infection of B-pathotype AYHR tetrad associated with a fifth RNA in individual root, and 3% for the A-pathotype AYPR tetrad. The A-pathotype tetrad AFHR, VYHR were found with a fith RNA for 0.43%, 0.21% respectively. Mixed BNYVV A-/B-pathotype infections with a fifth RNA were also observed in a individual sugar beet root. Tetrads found together in individual root like AYHR-AYPR, AYHR-AYHG, AYHR-AYPR-TYHR, VLH?-AFHR and AYHR-TYHR were found with a fifth RNA for 2.16%, 0.65%, 0.21%, 0.21% and 0.43% respectively. These possible reassortants account for 34% of the 482 samples with the B-pathotype AYHR being the most frequently found.

Table 2.3. Tetrad diversity observed in the Pithiviers region in France based on 482 isolates of *Beet necrotic yellow vein virus* (BNYVV) collected from 2008 to 2011.

BNYVV infection type	BNYVV type according to RNA-3	Data from Schirmer *et al.*, 2005		This study			Diversity in France
		RNA-3	RNA-5	RNA-3	RNA-5	Frequencies	Found in varieties (this study)
Single	B-type	**AYHR** 4	+	**AYHR** 4	+	26,45%	V1, V3, **V4**, V5, V6, **V7**, **V8**, **V9**, **V10**, **V12**, **V14**, V16, V18, V21, V23, V25, V26, V28, **V32**, V35
		AYHR 4	-	**AYHR** 4	-	25,05%	V1, V3, **V4**, V5, V6, **V8**, **V9**, **V10**, **V12**, V18, V19, V20, V21, V22, V30, (V33)
				TYHR	-	0,43%	**V8**
				AYHR 4 - TYHR	+	0,43%	V1, **V4**
				AYHR 4 - AYHT	-	0,21%	V5
	P-type	**SYHG** 4	+	**SYHG** 4	+	19,01%	V1, V2, V3, **V4**, V5, V6, **V9**, V16, V19, V20, V22, V25, V26, V28, (V33), V34, V36, V37
				SYHG 4	-	1,51%	V1, **V4**, V5
				SYHR	+	0,21%	V1
				SYHG 4 - SYHR	+	0,21%	V35
	A-type			**AYPR** 5	+	3,46%	**V4**, V5, V6, **V7**, **V10**, V17, V24, V27, V29, V30, V31
				AYPR 5	-	1,08%	V5, **V10**
		AFHR 2, 4	-	**AFHR** 2, 4	+	0,43%	V20
				VCHG 1, 3	-	0,43%	V1
				AYHG 2, 4	-	0,21%	**V4**
				AFPR	-	0,21%	V20
				VYHR	+	0,21%	**V4**
				VLH? 1 - AFHR	+	0,21%	**V4**
		ALHG	+	ND	ND	ND	Unknown
		AHHG	-	ND	ND	ND	Unknown
Mixed	B-/P-type			**AYHR** 4 - **SYHG** 4	+	9,93%	V1, V2, V3, **V4**, V6, **V7**, **V8**, **V9**, **V10**, (V33), V34, V37, V38
				AYHR 4 - **SYHG** 5	-	3,89%	**V4**, **V10**
				AYHR 4 - **SYHG** 4 -TYHR	+	0,21%	**V4**
	B-/A-type			**AYHR** 4 - **AYPR** 5	+	2,16%	V1, V6, **V10**, **V12**
				AYHR 4 - **AYPR** 5	-	0,65%	**V4**, **V10**
				AYHR 4 - **AYHG** 2, 4	+	0,21%	**V8**
				AYHR 4 - **AYHG** 2, 5	-	0,65%	V3, V5
				AYHR 4 - **AFHG** 2, 4	-	0,21%	**V10**
				AYHR 4 - **VCHG** 1, 3	-	0,21%	V1
				AYHR 4 - **AYPR** 5 -TYHR	+	0,21%	V1
				AYHR 4 - **AYHG** 2, 4 - **AYPR** 5	-	0,21%	**V10**
	P-/A-type			**SYHG** 4 - AFHR	+	0,21%	V20
				SYHG 4 - TFPR	-	0,21%	V39
				SYHG 4 - **AYPR** 5 - AFHR	-	0,21%	V39
	B-/P-/A-type			**AYHR** 4 - **SYHG** 4 - **AYPR** 5	+	0,65%	V6, **V10**
				AYHR 4 - **SYHG** 4 - **AYPR** 5	-	0,43%	**V10**

Tetrads observed also by other authors: **1.** Acosta-Leal *et al.*, Phytopathology 97: 325-330; **2.** Chiba *et al.*, J. Gen. Virol. 89: 1314-1323; **3.** Koenig *et al.*, J. Gen. Virol. 90: 759-763; **4.** Chiba *et al.*, Mol. Plant Microbe Interact. 24: 207-218; **5.** Bornemann and Varrelmann, Mol. Plant Pathol. 14: 356-364. Tetrads associated with resistance breaking (RB) in *Rz1* cultivars are in

bold and tetrads associated with RB in wild beet *B. vulgaris* subsp. *maritima* lines are under-lined.

NOTE. The frequencies established are based on these 482 sequences. For the tetrad VLH?, ? means that we weren't able to identify with certitude this aa because there were a lot of chromatographic peaks in the position 70 when direct sequencing was made. The cultivar in parentheses corresponds to a susceptible cultivar. The cultivars under-lined correspond to *Rz1Rz2* cultivars. The cultivars in bold correspond to *Rz1* cultivars with nematode resistance and the others are *Rz1* cultivar without the nematode resistance. ND means that we didn't found the tetrad in our study. The presence of RNA2 was also tested and was present for 63.73% of the samples. The occurrence of RNA5 with other tetrads than SYHG represents 33.77% of the samples. The total of mixed BNYVV pathotypes represents 20.25% of the samples and the total of single BNYVV pathotypes represents 79.75%.

BNYVV P-pathotype multiplied more in plant, but did not accumulate as much as B-pathotype in *Polymyxa betae* over the growing season. Based on field trials and comparison of 13 cultivars over 20 fields, both ELISA and RT-mqPCR targeting the BNYVV *p25* gene revealed a stronger accumulation of BNYVV P-pathotype than B-pathotype or mixed infections (Fig. 2.2 and Fig. 2.3). This observation was the same, regardless of the resistance background of the sugar beet cultivar. Despite this fact, the average increase of MPN over the growing season was stronger for B-pathotype infected soils as compared to P-pathotype or mixed pathotype soils, with a maximum multiplication factor of 230.77 compared to 14.44 or 104 times respectively (Fig. 2.4).

A striking feature of the field mapping was the diversity of virus pathotypes and tetrads observed in the fields (Fig. 2.2). Along with such diversity, changes observed between the situations early in the growing season and later on were also evident. For instance, it was the case for the fields DL09, BI10, CI10, CL11, GL11 and AI11. Specific tetrads not found in May were observed in September and vice versa. The number of zones per field where the virus was present also increased in September, but sometimes there were different tetrad/pathotype of virus. In general, the virus diversity tended to more tetrads at the end of the sugar beet season although this was not always the case.

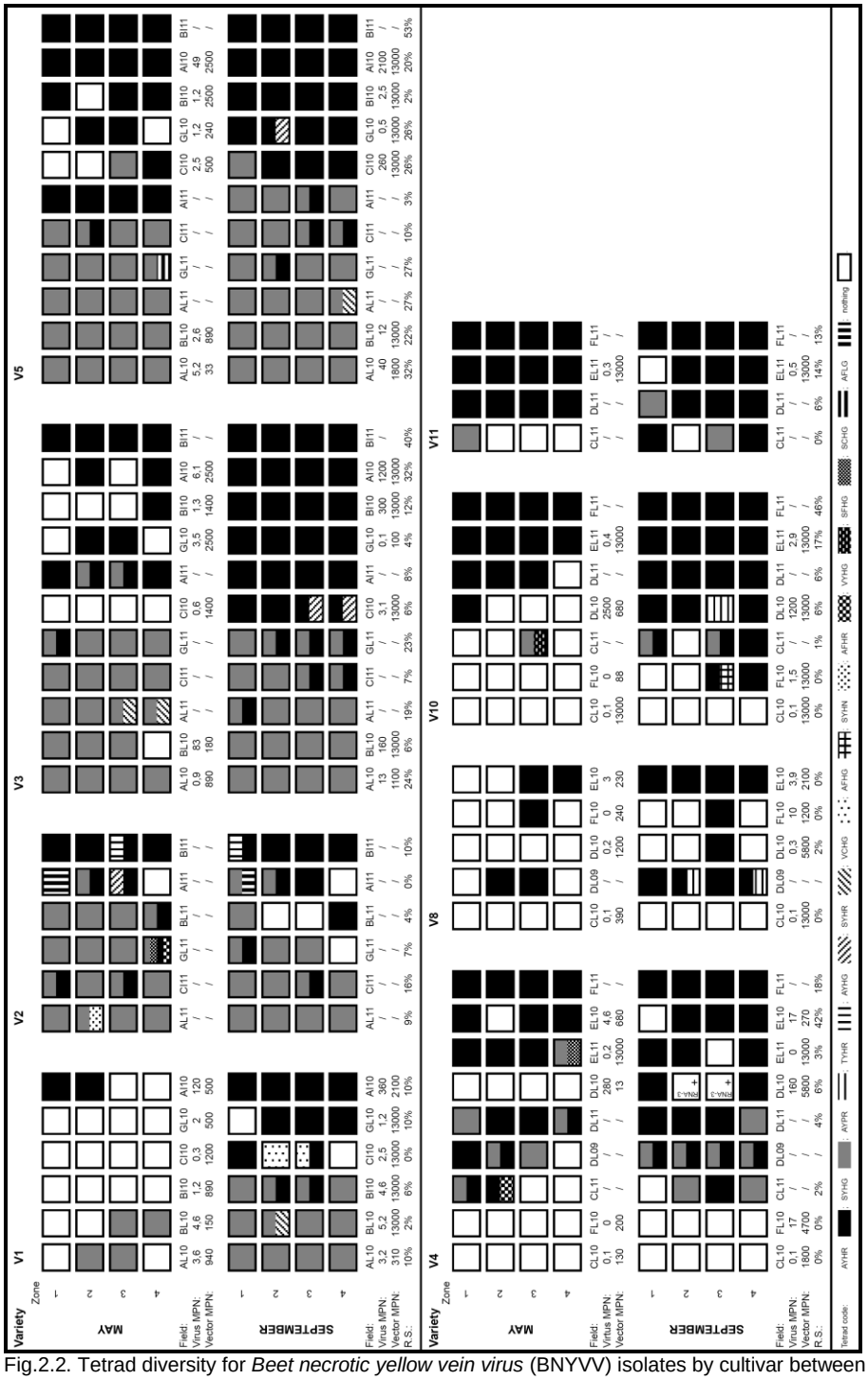

Fig.2.2. Tetrad diversity for *Beet necrotic yellow vein virus* (BNYVV) isolates by cultivar between 2009 and 2011 for samples collected from sugar beet fields in May and September in the region of Pithiviers of France. Each field was subdivided in four zones. The field code is reminded. The field EL09 and EL11 are the same field. V1 and V2 were *Rz1rz1Rz2rz2* cultivars. V3 and V5 to

V7 were *Rz1rz1* cultivars. V4 and V8 to V13 were *Rz1rz1* cultivars that also included tolerance to *Heterodera schachtii*. The Most Probable Number (MPN) mean values (infectious unit/g soil) for both virus and vector are provided. R.S. = root severity observed in September based on rhizomania symptoms. / = no information.

Mixed BNYVV pathotype and competition. Mixed infections of the collected sugar beet taproot were observed. In the field between 2009 and 2011, the tetrads AYHR and SYHG were the most frequently found in mixed infections. The three BNYVV pathotypes (A-pathotype, B-pathotype, P-pathotype) were also found in some samples. With the help of RT-mqPCR, the relative accumulation of B-pathotype (AYHR) and P-pathotype (SYHG) BNYVV RNA3 was measured. Based on the comparison of 46 field samples and seven cultivars, the BNYVV RNA3 content was systematically lower in case of mixed infection (AYHR and SYHG) than in the case of a single infection (Fig. 2.3). For SYHG alone, the highest *Rz1* is linked to 32% of root severity in the varietal band. The lowest *Rz1* is linked to 22% of root severity in the varietal band. For *Rz1*2, there is 10% of root severity and for *Rz1*S there is 6% of root severity in the varietal band. In mixed infection (AYHR and SYHG) with possible reassortment, the presence of the fifth RNA was also slightly detected in the RT-PCR. But in single infection with BNYVV P-pathotype associated SYHG, the fifth RNA5 was highly detected. For the highest quantitative values of single infection with the BNYVV B-pathotype associated AYHR, the fifth RNA was not detected. However, for the lowest values of this tetrad, the fifth RNA was detected in two cases. When there was mixed B- & P-pathotype infection, the recorded root severity was lower than for single infection (Table 2.4). In case of mixed infection, the observed

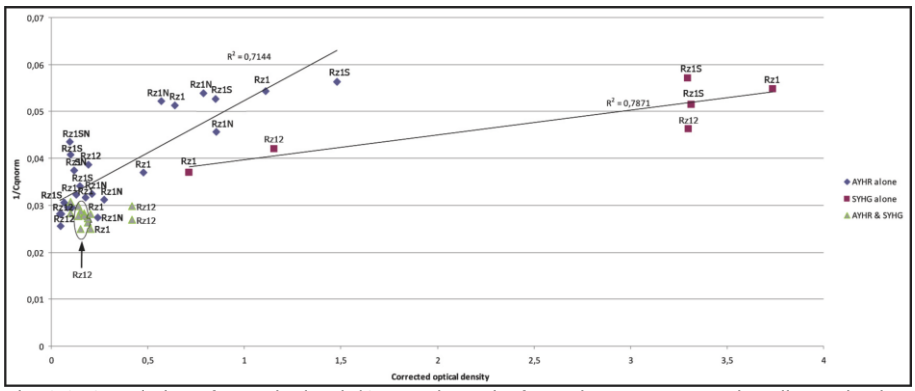

Fig. 2.3. Correlation of quantitative (1/Cqnorm) results for various *Beet necrotic yellow vein virus* (BNYVV) tetrads and enzyme linked immunosorbant assay (ELISA) values from 46 individual root plant samples collected in the Pithiviers region of France from a sugar beet field in September 2010. The tetrad combinations evaluated were SYHG alone (square), AYHR alone (diamond), and SYHG & AYHR in mixed infection (triangle). The Cq values were determined by RT-mqPCR with Taqman probes targeting the tetrad zone in the RNA3 of BNYVV genome and normalized with the ALS endogenous control gene in sugar beet. The corrected optical density values (three replicates minus twice the blank) are determined by DAS-ELISA targeting the CP protein coded by the RNA2 of the virus.
NOTE. *Rz1*S = *Rz1* cultivar with weak resistance to BNYVV, *Rz1* = *Rz1* cultivar with good resistance, *Rz1*SN = *Rz1* cultivar with weak resistance plus cyst nematode tolerance, *Rz1*N =

Rz1 cultivar with good resistance plus nematode tolerance, and *Rz1*2 = *Rz1Rz2* cultivar according to ITB classification.

root severity was the lowest for *Rz1rz1* cultivars with the nematode resistance, then with *Rz1rz1Rz2rz2* cultivars compared to *Rz1rz1* cultivars without nematode resistance. The P-pathotype SYHG tetrad alone was not detected in fields infested with nematodes, but was detected in mixed infections with other tetrads in these fields (Fig. 2.2). Overall, the P-pathotype was only present in 9.5% of the *Rz1rz1* cultivars with the nematode resistance compared to the other cultivars were this tetrad was present in 49.26%. Between the B-pathotype and the mixed pathotype, there were no significant differences among the ELISA values in cultivars with nematode resistance.

Heterogeneous and increasing virus/vector MPN. The heterogeneity of the infectious potential from different fields was assessed based on a bioassay with serial dilutions of rhizomania-infested soil for the measurement of the level of *P. betae* and BNYVV in the vector. The measured proportion of viruliferous vector (virus MPN/vector MPN) decreased over the growing season in average by 144% for 18 cultivars and increased in average by 3% for 12 cultivars on a total of 30 cultivars. However, the virus MPN (virus infectious potential) increased in the growing season in average by 2,857% for 24 cultivars, but decreased by 39% for six cultivars, and the vector MPN increased in average by 4,860% for 27 cultivars, but decreased by 74% for three cultivars. The vector MPN was much higher in the soil during the growing season than the virus MPN.

The virus was found in average in susceptible beet roots grown in soil dilutions between 1/125 and 1/3,215. *Polymyxa betae*, however, was found until soil dilutions of 1/78,125. The virus MPN in 2010 are shown in Fig. 2.2. Figure 2.4.A shows a large range for the multiplication factor (minimum and maximum) of the virus MPN in the soil during a sugar beet season when the BNYVV B-pathotype associated AYHR (-35 to 230.7) was detected in the plant samples. Only a statistically significant difference between the B-pathotype in May and September was observed (Fischer and Student test: *P = 0.05*) and no statistically significant difference between the BNYVV pathotype were observed in May and September. Between May and September, with regard to the P-pathotype, there was 2.39 times of increase of the mean MPN; with regard to the B-pathotype, there was 20.89 times of increase; and with regard to the mixed pathotype, there was 29.38 times of increase in the virus MPN.

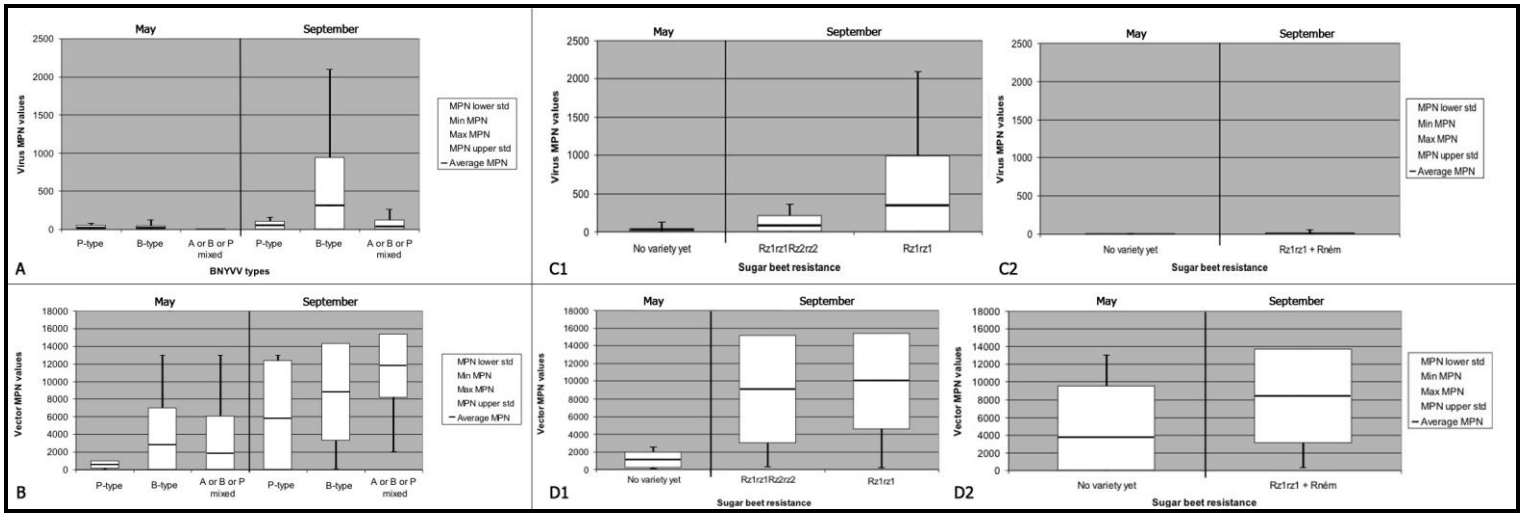

Fig. 2;4. Virus and vector Most Probable Number (MPN) in relation to *Beet necrotic yellow vein virus* (BNYVV) pathotype (P-, B-, and mixed pathotypes) and with the sugar beet resistance to BNYVV (*Rz1* and *Rz2*) and cyst nematode sampled in May and September. The virus MPN equals the number of viruliferous *Polymyxa betae* present in one gram of soil, while the vector MPN corresponds to the number of *P. betae* present in one gram of soil. The horizontal line represents the average MPN. The vertical line represents the Min-Max MPN. The box represents the upper and lower standard deviation around the MPN average. **Panel A.** Comparison of virus MPN values for May (before planting) and September (end of season). **Panel B.** Comparison of vector MPN values for May and September (end of season). **Panel C.1.** Comparison of virus MPN values for May and September based on BNYVV resistance source grown in fields without cyst nematode. **Panel C.2.** Comparison of virus MPN values for May and September based on BNYVV resistance source grown in fields with cyst nematode. **Panel D.1.** Comparison of vector MPN values for May and September based on BNYVV resistance source grown in fields without cyst nematode. **Panel D.2.** Comparison of vector MPN values for May and September based on BNYVV resistance source grown in fields with cyst nematode.

Table 2.4. Enzyme linked immunosorbent assay (ELISA) values and root severity for *Beet necrotic yellow vein virus* resistant cultivars grown in France.

Resistance background	BNYVV RNA3 pathotype	Mean ELISA ± SD	F-test	t-test		
Rz1rz1Rz2rz2	P B A or B or P mixed	1.75 ± 0.15 0.42 ± 0.29 0.63 ± 0.57	7.07 F theoric: 4.1	B-P: 2.96 t theoric: 2.2281	B-mix: 3.39	P-mix: 0.52
*Rz1rz1 without N	P B A or B or P mixed	1.81 ± 0.74 0.49 ± 0.24 1.04 ± 0.63	10.24 F theoric: 3.42	B-P: 4.50 t theoric: 2.0687	B-mix: 2.76	P-mix: 1.98
**Rz1rz1 with N	P B A or B or P mixed	Not observed 0.51 ± 0.23 0.46 ± 0.14	0.24 F theoric: 4.41			
		Mean root severity ± SD				
Rz1rz1Rz2rz2	/	7% ± 4.80%	5.80 F theoric: 3.17	Rz2-Rz1 without nematode: 2.91 t theoric: 2.0059	Rz1 without nematode-Rz1 with nematode: 0.53	Rz2-Rz1 with nematode: 2.76
*Rz1rz1 without N	/	19.12% ± 12.82%				
**Rz1rz1 with N	/	9.30% ± 13.19%				
Rz1rz1Rz2rz2 *Rz1rz1 without N **Rz1rz1 with N	P P P	9.5% ± 0.71% 19% ± 9.34% not observed	1.86 F theoric: 5.99	/		
Rz1rz1Rz2rz2 *Rz1rz1 without N **Rz1rz1 with N	B B B	10% ± 0% 21.37% ± 18.54% 12.85% ± 15.27%	0.82 F theoric: 3.49	/		
Rz1rz1Rz2rz2 *Rz1rz1 without N **Rz1rz1 with N	A or B or P mixed A or B or P mixed A or B or P mixed	5.62% ± 5.54% 17.4% ± 9.81% 2.71% ± 2.63%	10.51 F theoric: 3.44	Rz2-Rz1: 3.48 t theoric: 2.0739	Rz1-Rz1nem: 4.18	Rz2-Rz1nem: 0.79

The average ELISA values are represented as the average optical density of the four zones (three replicates per zone minus twice the blank) more or less a standard deviation (SD). The average of the average ELISA values is then calculated for each pathotype within each type of resistance. There is a statistically significant difference between the comparisons among the three different categories of means (type of resistance) at $P = 0.05$ when F obs > F test. After that, a student test (t-test) was made for side-by-side comparison to know between which subcategories (pathotype) were different. There is a statistically significant difference when t obs > t test. The same type of calculation was done for the average root severity.
NOTE. * *Rz1rz1* cultivars without N (nematode resistance); ** *Rz1rz1* cultivars with N (nematode resistance).

The Figure 2.4.B shows the same for the vector MPN but in a higher level. However, when the BNYVV P-pathotype associated SYHG was found in the samples, the virus MPN was very low suggesting a weak relationship of this tetrad with the vector. The multiplication factor for the P-pathotype was comprised between -1.12 and 14.44. For the mixed infection of the three BNYVV pathotypes (A-pathotype or B-pathotype or P-pathotype), the range for the multiplication factor was intermediate (comprised between -2.4 and 104) and we observed only high virus MPN when there was mixed infection with at least the tetrad AYHR. During the sugar beet season, the virus MPN increased mainly when the B-pathotype was detected in the

plant. However, the vector MPN increased for each pathotype. During the sugar beet season, the multiplication factor of the vector MPN for the P-pathotype, the B-pathotype and the mixed pathotype was comprised between -3.03 and 72.22, -25 and 446.15 and, 0 and 147.73 respectively. There were statistically significant difference (*P = 0.05*) in each pathotype between May and September and no statistically significant difference between the BNYVV pathotype were observed in May and September. Between May and September, with regard to the P-pathotype, there was 9.96 times of increase of the mean MPN; with regard to the B-pathotype, there was 3.16 times of increase; and with regard to the mixed pathotype, there was 6.22 times of increase in the vector MPN.

When comparing MPN versus the source of resistance, we observed very low virus MPN with the *Rz1Rz2* cultivars. The virus MPN was higher in *Rz1* cultivars (Fig. 2.4.C.1). In general, the virus MPN increased during the sugar beet season in *Rz1* and *Rz1Rz2* cultivars. With the *Rz1rz1* cultivars with cyst nematode resistance, the virus MPN didn't increase (Fig. 2.4.C.2). During a sugar beet season, the multiplication factor of the virus MPN for the *Rz1rz1Rz2rz2*, *Rz1rz1* without cyst nematode resistance and *Rz1rz1* with cyst nematode resistance was comprised between -1.67 and 8.33, -35 and 230.77 and, -2.08 and 20.48, respectively. The increase for the virus MPN was the highest for the *Rz1rz1* cultivars without cyst nematode resistance. There is only a statistically significant difference between May and September for the *Rz1rz1* cultivars without cyst nematode resistance (*P = 0.05*) and no statistically significant difference between the cultivars were observed in May and September. Between May and September, with regard to the *Rz1Rz2* cultivars, there was 3.58 times of increase and with regard to the *Rz1* cultivars without resistance to cyst nematode, there was 19.46 times of increase. With regard to the *Rz1* cultivars with resistance to cyst nematode, there was 8.8 times of increase.

For the vector MPN, we observed high MPN in the three different varieties (Fig. 2.4.D.1 and 2.4.D.2). The vector MPN increased during the sugar beet season in each variety. During the season, the multiplication factor of the vector MPN for the *Rz1rz1Rz2rz2*, *Rz1rz1* without nematode resistance and *Rz1rz1* cultivars with nematode resistance was comprised between -3.03 and 86.67, -25 and 72.22 and, -2.52 and 446.15, respectively. Consequently, the *Rz1rz1* cultivars with nematode resistance increase vector number but not the virus titer. There is an intrinsic resistance in these cultivars towards the virus itself rather than the vector. There is a statistically significant difference between May and September for each cultivar (*P = 0.05*) and no statistically significant difference between the cultivars were observed in May and September. Between May and September, with regard to the *Rz1Rz2* cultivars, there was 8.28 times of increase and with regard to the *Rz1* cultivars without resistance to cyst nematode, there was 9.13 times of increase. With regard to the *Rz1* cultivars with resistance to cyst nematode, there was 2.25 times of increase.

Presence or absence of BSBV, BVQ, BBSV or BSBMV. No BBSV and BSBMV were detected in France, as expected based on knowledge of their

distribution. The virus detection level was highest (79%) with BNYVV (RNA3), followed by BVQ (67%) and BSBV (62%).

BSBV controlled by cultivars with *Rz1* and *Rz2*. Between 2009 and 2011, different cultivars were grown in field assays in the region of Pithiviers. The results showed that BSBV control was influenced by the *Rz2* resistance gene. In most cases, BSBV was much lower in cultivars with *Rz1rz1Rz2rz2*, while, in the same fields *Rz1rz1* cultivars were infected by BSBV. In general, the root severity and the incidence of the rhizomania disease were also much lower with these *Rz1rz1Rz2rz2* cultivars such as V2 and V1. BSBV incidence was 50 to 70% less with cultivars containing both *Rz1* and *Rz2*, while the cultivars with only *Rz1* were all infected by BSBV.

Discussion

The epidemic foci located in the Pithiviers region, south of Paris in France was much different from resistance-breaking described in other countries (Rush *et al.*, 2006; Koenig *et al.*, 2008 and 2009b; Acosta-Leal *et al.*, 2010; Chiba *et al.*, 2011). As Schirmer *et al.* (2005) already pointed out the presence of various BNYVV pathotypes and tetrads, this study emphasizes the diversity both at the area and field level, hence underlining the complexity of the disease.

The situation in Pithiviers is different from that in other sugar beet production regions. A-, B- and P-pathotypes are all present in the Pithiviers region, which makes it quite uniques. The strong selection pressure created by widespread use of resistant cultivars seems to favor the occurrence of parallel mutations in individual virus populations, calling into question how long *Rz1* resistance will be useful (Acosta-Leal *et al.*, 2010; Bornemann and Varrelmann, 2013). Chiba *et al.* (2011) have proposed an evolutionary pathway reconstructing the spread and the occurrence of resistance breaking isolates of BNYVV in different countries (VCHG, VLHG, ALHG, AHHG, ACHG, AFHG, SYHG, AQHG, AYRV). The resistant-breaking (RB) selection can be found in association with only one aa mutation in the tetrad (Bornemann and Varrelmann, 2013) and also other aa positions in the p25. Usually, both the WT and RB tetrad are geographically localized. In the USA, a RB zone without a fifth RNA is linked with a single mutation in the *p25* gene (Acosta-Leal *et al.*, 2010).

Rhizomania in France around Pithiviers was much different from the other regions with resistant-breaking in the USA, Europe (Acosta-Leal and Rush, 2007; McGrann *et al.*, 2009), and Asia (Chiba *et al.*, 2011), because much of the known world virus diversity can be found in only a 150 km^2 in production area. Nine recognized and putative (SYHG, AYHG, AFHG, AFHR, AYPR, VLHG, VCHG, TYHR and AYHR) *Rz1*- or *Rz2*-RB BNYVV isolates have been documented in the area. Furthermore, the simultaneous presence of at least three BNYVV pathotypes along with evidences for reassortments (Koenig *et al.*, 2008) between isolates highlights the need to manage as efficiently as possible the sources of resistance available. Peltier

et al. (2008) also found RNA5 associated with European BNYVV A-, B- and SYHG P-pathotypes (Peltier *et al.*, 2008).

Spatial diversity at regional and field level. Besides these observations, a striking feature is also the high frequencies (2009-2011) of BNYVV P-pathotype (SYHG tetrad) or BNYVV B-pathotype (AYHR tetrad) as compared to the very low frequencies measured for known BNYVV A-pathotype RB isolates with the tetrads VCHG or others. Based on our field-based trial approach, it was possible to determine that the diversity was found not only at the regional level but also at the field-scale level, and both at the beginning and at the end of the growing season. Using deep sequencing and bioassays, Bornemann and Varrelmann (2013) found that sugar beet genotype has a strong selective effect on the accumulation of different p25 tetrads, and that RB tetrad mutations are selected with a loss of relative fitness, with the exception of P-pathotype. Some genotypes would select for the development of viral strains and other genotypes would support the development of other unrelated specific viral strains. The diversity observed in the Pithiviers area could be a reflection of the major breeding and rhizomania resistance research effot that has taken place in the area since 1970's (De biaggi *et al.*, 2003).

In general also, intra-field diversity with inter-variety behavioral divergence was also observed. Before 2005, Schirmer *et al.* (2005) found in France three pathotypes and five tetrads known in the area, based on 40 isolates. There were the BNYVV B-pathotype associated AYHR (54%), followed by BNYVV P-pathotype associated SYHG (32%) and BNYVV A-pathotype associated AFHR (3%), AHHG (3%) and ALHG (6%) (Table 3.3). According to Bornemann and Varrelmann (2013), different sugar beet genotypes would support the development of divergent viral populations. In contrast to the 2005 results of Schirmer and colleagues, we found greater tetrad diversity in each pathotype, i.e. in B-pathotype, AYHR, TYHR, AYHT, AYHS and ASHR, in P-pathotype, SYHG, SYHR, SYHN, SFHG and SCHG, and in A-pathotype, AYPR, AYHG, AFHG, AFHR, AFLG, AFPR, VCHG, VYHG, VLH?, VYHR and TFPR (Table 2.3). The tetrad AYPR was also associated here with clear resistance-breaking events. Several tetrads usually associated with the A-pathotype or the B-pathotype were also found with the fifth RNA.

BNYVV P-pathotype accumulates within the single sugar beet roots and is in competition with BNYVV B-pathotype. In 2006, Doucet *et al.* have showed that there was a virus heterogeneity and distribution in a single rootlet and that there is a relative homogeneity of *P. betae* in the beet rootlet. Moreover, it has been showed that the P-pathotype of BNYVV is contained in the central root stele and the B-pathotype of BNYVV is localized in the peripheral cortical layer subepidermal. In the present study, the BNYVV RNA5 was detected in 41% of the 1,058 samples analyzed. The relationship between the tetrad SYHG related to the P-pathotype and agressiveness of the disease and the sensitivity is still not clear. When compared by ELISA, BNYVV P-pathotype accumulates at much higher levels in both *Rz1* cultivars with good resistance, *Rz1* cultivars with weak resistance and susceptible

sugar beet varieties than BNYVV B-pathotype. Therefore, this suggests that the P-pathotype could move more easily in the plant than the B-pathotype. Surprisingly, such quantification and quantitative RT-PCR have shown a competition between both pathotypes when detecting mixed infection within a single beet. The BNYVV mixed infection level was lower than in P-pathotype single infection level or B-pathotype single infection level. When there were mixed infection of the two tetrads AYHR and SYHG in a single sugar beet, the CP (RNA2) content was lower in the plant than in single infection of each tetrad. It seems that there is a modulation of the different BNYVV RNAs in the plant according to the pathotype present. The tetrad SYHG was also linked to very high level of ELISA values and consequently in CP content. However, the tetrad AYHR was linked to lower content of CP in the plant but higher in case of mixed infection. The competition between B- & P-pathotype is less evident when comparing the ELISA data. It seems that the two tetrads are in competition in a single sugar beet taproot. There were no correlation between the root severity and the ELISA values (data not shown). The competition between the tetrad AYHR and SYHG, the two most present tetrads observed in the fields, was more observed rather than reassortment or recombination. Perhaps, it is a prerequisite before the emergence of reassortants.

Tetrad usually associated to B-pathotype or A-pathotype with a fifth RNA. A prerequisite to recombination or reassortment is the presence of the different pathotypes in the same cell of a plant. Meunier *et al.* (2005) suggested that a recombination event had taken place within the RNA2 between the BNYVV B-pathotype and A-pathotype (Meunier *et al*., 2005). In 2009, Koenig *et al.* suggested BNYVV genome reassortment in several resistant sugar beet varieties with strong rhizomania symptoms (Koenig *et al*., 2009a). The fifth RNA was detected either with A-pathotype RNAs 1 to 4 or with a mixture of B-pathotype and P-pathotype RNAs. But, the distinction between mixed BNYVV pathotype infection and reassortment was not clear. In Spain, particular tetrads (VLHG) linked to the BNYVV A-pathotype were responsible of resistance-breaking and the fifth RNA was not found (Koenig *et al*., 2005c). In Italy, only the A-pathotype without the fifth RNA was found (Koenig and Lennefors, 2000; Marc-Richard Molard, Institut Technique de la Betterave (ITB), personal communication). Gilmer *et al.* have already demonstrated that B-pathotype RNA1 and RNA2 with the fifth RNA replicon worked very well. In this study, mixed infections of tetrads were related to the A-pathotype and/or B-pathotype and/or P-pathotype in a single sugar beet taproot but not in a single cell. Approximately 20% had a fifth RNA with a tetrad associated to the A-pathotype and/or B-pathotype. This indicates a stronger presence of A-pathotype or B-pathotype containing P-pathotype RNA5 “reassortants” than previously indicated (Koenig *et al.*, 2009a).

Increase of infectious potential of BNYVV in the soil over the growing season depends on resistance background. Conversely to the decrease in infectious potential described by Lennefors *et al.* (2006) with highly resistant hybrids, an increase of the inoculum level was monitoring in this study. The hybrid susceptible to BNYVV observed by Lennefors had

increased the inoculum level. Tuitert *et al.* (1994) showed that the infectious potential of BNYVV (the most probable number of viruliferous *P. betae* propagules) in soil (a field trial sampled in 1991) was lower after growing a resistant cultivar than after growing a susceptible cultivar. In contrast, at four sites sampled in 1990, infectious potential was only slightly increased by growing sugar beet and was not significantly affected (*P = 0.05*) by the cultivar grown. In resistant plants, which have low virus content in the roots, the populations of resting spores formed are less viruliferous than that in the susceptible plants with high virus content (Tuitert *et al.*, 1994). At the field level, this study shows the level of virus inoculum increase taking place between the beginning and the end of the season, depending on the level of resistance of the sugar beet varieties used. On average, the virus inoculum increase was of 29.5 times and the vector inoculum increase was of 35.1 times. In the present study, the vector MPN was much higher than in results of Goffart *et al.* (1987), but the detection sensivity are also currently more efficient. Water availability during the season was supplied in each field by irrigation. The statistical virus MPN mean comparison between *Rz1Rz2* cultivars, *Rz1* cultivars tolerant to nematode and *Rz1* cultivars not tolerant to nematode showed different behaviors. The *Rz1Rz2* cultivars allowed an increase of 3.58 times in average and the *Rz1* cultivars without the nematode resistance allowed a higher increase of 19.46 times. This variety showed also an increase in the virus MPN during a sugar beet season when the samples were infected by the tetrad AYHR. When the tetrad AYHR was detected in the different field and independently of the cultivars, there was an increase of 29.36 times of the virus MPN in the soil. However, *Rz1* cultivars with nematode resistance showed an increase of the virus MPN of only 8.8 times in the field during the sugar beet season. But, the virus MPN was much lower in this variety compared to the other cultivars. Although by now there is no clear explanation of the reason of such an effect except a synergistic effect of nematodes triggering the rhizomania process, possibly by favoring the vector *Polymyxa betae* or by weakening plant resistance. Cultivars with *Rz1* and nematode resistance gene should be interesting for a possible virus inoculum decrease over years of rotation. One field with tetrad (A-, B- and P-pathotype) diversity showed a decrease of the virus inoculum in the soil during a single sugar beet season with cultivars containing the *Rz1* resistance gene and nematode resistance.

In this study, there are variable and contrasted values of *P. betae* and BNYVV virus in the selected soils. The proportion between viruliferous and non-viruliferous *P. betae* showed a weak concentration of viruliferous *P. betae* in the field. For comparison, in Belgian soils, the proportion of total vector that are viruliferous vector (= virus MPN/vector MPN) is around 20%. Ciafardini has used standards in 1991 to show the proportion of viruliferous vector to the total germinating vector present in the soil could range from 11 to 44% (Ciafardini *et al*., 1991). The soil samples originated from Northern Italy, England, Austria and USA (Ciafardini and Marotta, 1989). They determinated that 28% of the vector population was viruliferous, while the proportion in debris samples was 20%. Goffart *et al.* (1987) described weak infectious potentials (vector MPN) in a range between 0.01 to 13.6 infectious units (IU) per gram of soil (20 repetitions per dilution). Goffart *et al.*

considered high vector infectious potential when the IU/gr of soil was higher than 13.6. The higher vector MPN observed in 1990 in Belgium was 123.3 IU/gr of soil. On 52 different fields studied in Belgium by Goffart *et al.*, the majority of all the sugar plants infected by *Polymyxa betae* were no more infected in the 1/3,125 soil dilution than they were after the 1/78,125 dilution in France between 2009 and 2011.

In France, soils from fields cultivated with cultivars containing *Rz1* were observed to have both high BNYVV and vector levels. It would seem that *Rz1* resistance has partly eroded, but this was not the case in the USA (Rush *et al.*, 2006). Consequently, some soils in Europe might show high inoculum density of viruliferous *P. betae*. The content of P-pathotype in the tap root of sugar beet is also more important than A- or B-pathotype infections indicating that the P-pathotype moves more rapidly in the plants than the two other BNYVV pathotypes (Heijbroek *et al.*, 1999). This relationship was also observed in the present study. Other authors pretend that RB isolates are independent of the inoculum density of the vector and the virus and the independent geographically RB selection follow the culture of *Rz1* sugar beet (Pferdmenges *et al.*, 2009; Bornemann and Varrelmann, 2013). In our study in 2010, the B-pathotype present in the root samples was linked to high virus MPN in the soil. It was not the case for the P-pathotype or mixed infection of pathotype. And, the virus MPN increased more in *Rz1* cultivars without nematode resistance than in *Rz1Rz2* cultivars. The BNYVV P-pathotype has a poor relation with its vector and the virus compensates this weakness in increasing its relationship with the sugar beet. Indeed, the P-pathotype had a higher titer level in the taproot than the B-pathotype. Competition between the tetrad related to the B-pathotype and P-pathotype was evident. The proportion of viruliferous vector (virus MPN divided by vector MPN) contrasted between 0% and 26% but it was not always related to the visual rhizomania symptoms. The bio-assay tetrad results were different from the results obtained in the field where the sugar beet plants were older. The tetrads identified in the bio-assays were not so diversified than the tetrads identified in the field but they were more homogeneous and uniform. The main tetrad was revealed. Of course, the temperature and growing conditions in the field and in the bioassay are completely different. But, the bio-assay tetrads were in relation with the reality observed in term of distribution in the field. Overall, MPN for both virus and vector were weaker in May 2010 compared to September 2010. There was an increase of the BNYVV and vector inoculum in the soil due to the presence of sugar beet in the field even when the proportion of viruliferous vector decreases. This means that the decrease of the proportion of viruliferous vector was due especially to the high increase of the vector MPN.

Presence of BSBV and BVQ in France and BSBV control by *Rz2* varieties. Prillwitz and Schlösser (1993) showed that initial infection with BSBV results in reduced disease severity upon subsequent infection with BNYVV, in comparison with sugar beet plants infected only with BNYVV (Prillwiz and Schlösser, 1993). The possible involvement of BSBV and BVQ in the epidemiology of rhizomania disease is not yet well elucidated (Heidel

and Rush, 1997; Meunier *et al.*, 2003, Pavli *et al.*, 2010). Besides the frequent mixed BNYVV pathotype field infections in our study in France, BNYVV was often found together with the pomoviruses *Beet virus Q* and *Beet soil-borne virus*. BSBV and BVQ have also been found in single and mixed infections with BNYVV in single sugar beet roots. Thus, there is the potential for interaction and complementation between BSBV, BVQ and BNYVV due to the mixed infection (Crutzen *et al.*, 2010). Indeed, BVQ might not multiply without BNYVV and there might have cross-protection against BNYVV when BSBV pre-infects the sugar beet (Crutzen *et al.*, 2010). We could suppose that there is firstly a competition between the pomoviruses and BNYVV and lastly a competition between the different BNYVV pathotypes. Their role in the rhizomania syndrome remains a matter of debate, but attention should be given to the possibly of competition taking place at the beginning of the growing season, when the soil temperature is lower and the pomoviruses with their lower temperature optimum might have a comparative advantage. It should also be noted that no other sugar beet soil-borne viruses like *Beet black scorch virus* or *Beet soil-borne mosaic virus* were detected (Gonzalez-Vasquez *et al.*, 2009).

A synergism and/or competition between the various soilborne viruses might have been present. The incidence of BVQ may varied from 30% of the tested samples in Belgium (Stas *et al.*, 2001a) to 1.5% in Iranian ones (Farzadfar *et al.*, 2005) and was always lower to that of BSBV, which was reported to be 85% and 9.5% of the tested fields for the same countries, respectively. However, in the present study, the level of BNYVV (RNA3) in the samples collected was the highest (79%), followed by BVQ (67%) and BSBV with (62%). These high proportions of BNYVV and *Pomovirus* associated detected in the samples can be linked to the high level of *Polymyxa betae* in the French soils (high level of MPN). These numbers correspond also to the virus presence in field assays conducted with *Rz1rz1Rz2rz2* and *Rz1rz1* cultivars. Besides, the cultivars with both *Rz1* and *Rz2* resistance genes led to a reduction of 50 to 70% in BSBV content and almost no symptoms. With Pithiviers samples in general, a large proportion of the samples had three viruses in simple or mixed infection. The incidence of BSBV and BVQ was found at a lower level in other countries like Iran, Belgium and USA. It's known that BSBV prolifers independently from BNYVV, but BVQ is not able to multiply without BNYVV (Crutzen, 2010).

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CHAPTER 3

Micro-evolution of BNYVV

3.1 Short introduction

This second paper, which will be published in *Virus Research*, has been selected following an oral presentation at the International Plant Virus Epidemiology Conference, held in Arusha, Tanzania in January 2013. We hypothesize that the sugar beet resistance profile used can influence the virus evolution through micro-evolution. We estimate that the resistance genes push the viral population to diversify itself. There is a selection pressure of the plant on the virus. To keep its fitness, the virus must adapt itself with genetic change in the genes involved in the virus-host interaction. Consequently, we analyzed the impact of several sugar beet resistance genes on the BNYVV genome through NGS. As a function of the resistance profile of the beet plants, we tested if the virus is able to induce an infection and if it is the case, we try to detect the changes in the virus genome.

3.2 Project paper in the journal "*Virus Research*"

Within-host evolution of *Beet necrotic yellow vein virus* during a single sugar beet crop season

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Abstract

Within-host evolution of *Beet necrotic yellow vein virus* (BNYVV) was studied using sugar beet with different levels of virus resistance in the Pithiviers area south of Paris in France. The virus distribution in the experimental field was mapped at the beginning and end of the season, with samples from the experimental field and from bioassays. Irrigation was used during the season in the field assay. To understand the impact of the sugar beet cultivars on the evolution of BNYVV, 454 deep-sequencing was used to generate sequences from BNYVV RNA2, RNA3 and RNA5 targeted amplicons. The samples were collected in a field plot where the disease distribution had been characterized. The predominant pathotype was the BNYVV P-pathotype with the SYHG tetrad and the fifth genomic RNA. The rhizomania-associated soil-borne viruses (BSBV and BVQ) were also in evidence. Three partially resistant cultivars were used with different BNYVV resistance backgrounds: *Rz1* cultivars with weak resistance (*Rz1rz1Rz2rz2*), *Rz1* cultivars with good resistance (*Rz1rz1Rz2rz2*) and cultivars with two partial resistance genes (*Rz1rz1Rz2rz2*). The estimated diseased root impact in September varied from 2% in the *Rz1Rz2* cultivars to 6% in the *Rz1* cultivars and 22% in the *Rz1* cultivars with weak resistance. Amplicons were obtained using tagged primers positioned in conserved BNYVV genome regions. The genes targeted were thought to be involved in the interaction with the plant virus: *p25* of BNYVV RNA3, *p14* of BNYVV RNA2 and *p26* of BNYVV RNA5. The 454 deep-sequences were analyzed for all samples. About 6000-7000 sequences per sample for each amplicon/ cultivar combination were obtained. The analysis of conventional sequences obtained at the beginning and end of the sugar beet growing season, together with sequences from bioassays using a susceptible (*rz1rz1rz2rz2*) cultivar as well as deep-sequencing, showed variations depending on the sugar beet genotype and the spatial dispersion of the virus. The mutation and the mutation frequency in the acid nucleic position of each gene differed totally in the different

cultivars, although the experimental field was a homogeneous one, infected with P-pathotype of BNYVV.

Keywords
Rhizomania, 454 deep-sequencing, sugar beet cultivars, virus diversity, resistance breaking isolates, BNYVV, Polymyxa betae

1. Introduction

Beet necrotic yellow vein virus (BNYVV), a *Benyvirus*, is the causal agent of rhizomania, a major disease of sugar beet. First discovered in the late 1950s, the disease is now widely distributed across the world (Tamada & Baba, 1973; Richard-Molard, 1985; Meunier *et al.*, 2003; Rush *et al.*, 2003, 2006; Schirmer *et al.*, 2005; Chiba *et al.,* 2008, Mehrvar *et al.*, 2009; Peltier *et al.*, 2008). Typical symptoms are shown in Figure 3.1. The BNYVV genome consists of four single-stranded RNA molecules, sometimes with an additional RNA (Tamada *et al.*, 1989; Hleilbieh *et al.*, 2007; Covelli *et al.*, 2009; McGrann *et al.*, 2009). Kruse *et al.* (1994) proposed grouping BNYVV into different pathotypes (A, B, P) based on restriction fragment length polymorphism (RFLP) profiles (Koenig *et al.*, 1995a). More recently, based on phylogenetic analysis, Schirmer *et al.* (2005) proposed another pathotype called J.
In recent decades, the use of resistant sugar beet cultivars has helped to control the virus (Lewellen *et al*., 1987; Pelsy & Merdinoglu, 1996; Scholten *et al*., 1996). The distribution of high-yielding and resistant cultivars contributed to the rapid and wide distribution of *Rz1* resistance (Biancardi *et al*., 2002). Resistance events and resistance-breaking isolates able to overcome the host's resistance were soon reported (Liu *et al*., 2005; Acosta-Leal *et al.*, 2010; Pavli *et al.*, 2011). Cultivars with two genes for resistance (*Rz1rz1*, *Rz2rz2*), however, showed a considerably lower virus titer than the susceptible and *Rz1* cultivars with weak resistance check, suggesting that the additional *Rz2* resistant gene gave partial resistance against BNYVV (Rush *et al.*, 2006).

Koenig *et al.* (1995) initially described the stability of the viral genome, except for BNYVV RNA3. Several studies (Meunier *et al.*, 2005; Mehrvar *et al.*, 2009; Schirmer *et al.*, 2005; Koenig *et al.*, 1995a, 1995b; Koenig and Lennefors, 2000) showed that the BNYVV genome is relatively stable compared with other viruses, with only a limited number of changes, except probably for RNA3 (*p25*). Despite this, Schirmer *et al.* (2005) showed the presence of a hot spot for mutations within the *p25* gene (amino acids [aa] positions 67 to 70), subjected to positive selection pressure. It was hypothesized that this 'tetrad' was involved in the resistance breaking (Pferdmenges *et al.*, 2009). Koenig *et al.* (2009) showed that a single U/C nucleotide substitution changing alanine to valine in the p25 protein promoted increased virus accumulation in the roots. Acosta-Leal *et al.* (2010) also showed that specific single nucleotide changes could affect the plant's resistance.

Fig. 3.1. Rhizomania symptoms. Most of the pictures were taken in the Pithiviers region. Foliar symptoms: a. Systemic foliar symptoms with yellowing and necrosis of the leaf veins; b. Foliar wilt of infected sugar beets; c. Yellowing leaves in rows of susceptible sugar beets (cultivar 'Encarta'). Root symptoms: d. Vascular system browning and lignification (vb); e. Proliferation of lateral rootlets (taproot as beard-like appearance); f. Root constriction (rc); g. Reduction in the taproot size of *Rz1* cultivars with weak resistance (right) compared with *Rz1* cultivars (left); h. *Polymyxa betae* sporosores.

Nevertheless, resistance-breaking events are not always related to valine-alanine substitution (Rush *et al.*, 2006), as in the UK where the A-pathotype associated AYPR tetrads were responsible for symptoms (Mark Stevens, IIRB meeting, Broom's Barn UK, 2011) and the SYHG and TYHG tetrads were sometimes observed with the fifth RNA (Harju *et al.*, 2002; Ward *et al.*, 2007), or as in the area south of Paris, in France, a region with a long history of rhizomania.

In our study, the hypothesis of within-host evolution during a single sugar beet crop season was tested in order to understand and clarify virus genome micro-evolution (i.e., small genetic changes below the species level). The

micro-evolution of RNA viruses, especially in the guise of molecular epidemiology, shows how various evolutionary processes have shaped intra-species and intra-host genetic diversity (Conway and Roper, 2000; Kitchen *et al.*, 2011). Micro-evolution has been reported for human viruses such as HIV and hepatitis C. Fischer *et al.* (2010) revealed the dynamics of early immune escape following a single HIV-1 transmission by ultra-deep sequencing. Campbell *et al.* (2011) studied viral linkage in HIV-1 seroconverters and their partners in a clinical HIV-1 prevention trial throughout pyrosequencing (Langaee and Ronaghi, 2005). Wang *et al.* (2007) detected an average of 58 variants of HIV per sample compared with an average of eight variants per sample detected by conventional direct-PCR dideoxynucleotide sequencing. Delang *et al.* (2011) conducted a comparative study of the genetic barriers and pathways in the resistance of selective inhibitors of Hepatitis C virus replication.

To date, most commercial sugar beet cultivars used in rhizomania-infested areas are based on using the *Rz1* gene combined with minor genes. An important tetrad diversity in the Pithiviers area had been observed (Galein *et al.*, submitted for publication). We wondered if such diversity was due to the cultivar grown in the field and if the cultivar could influence the virus population. Several field assays were conducted between 2009 and 2011 to see if the varietal pressure in one sugar beet season could produce a virus evolution. Some of the fields (heterogeneous and homogeneous) studied in collaboration with the Institut Technique de recherche sur la Betterave (ITB) are represented in Figure 3.2. This study was set up within the framework of a cooperative research approach developed with the French sugar beet technical institute with the aim of testing the hypothesis that the virus could micro-evolve within a single crop rotation, depending on varietal genetic background. To reflect real field conditions, the materials used in this research were obtained from one homogeneous trial plot set up in 2010. Deep-sequencing was used to obtain the highest diversity in a single sugar beet root and in three cultivars (*Rz1rz1Rz2rz2* cultivars with weak resistance, *Rz1rz1Rz2rz2* cultivars and *Rz1rz1Rz2rz2* cultivars) with different resistance backgrounds.

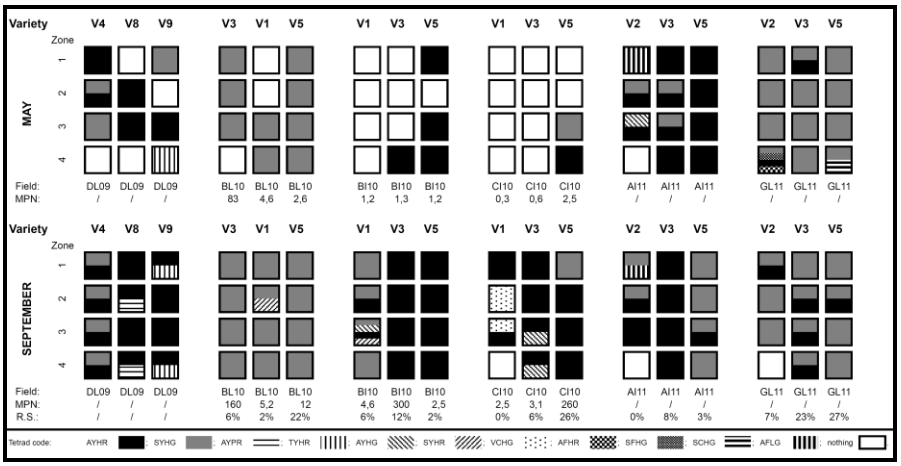

Fig. 3.2. Representation of the tetrad diversity per cultivar in several fields in 2009, 2010 and 2011 from the start (May) or end (September) of the growing season in the Pithiviers region in France. Each field was subdivided into four zones. Each year, there were 8 to 10 fields: 2009: DL09 - Sougy 1; 2010: BL10 - Yèvres-la-Ville 1, BI10 - Chenou 2, CI10 - Gironville 1; 2011: GL11 - Yèvres-le-Chatel 2 and AI11 - Mondreville 3. The genetic background differed among each cultivar. V1 and V2 were *Rz1rz1Rz2rz2* cultivars. V3 and V4 were *Rz1rz1* cultivars. V4 was also *Heterodera schachtii*-tolerant. V5 was a *Rz1rz1* cultivar. V8 and V9 were *Heterodera schachtii*-tolerant *Rz1rz1* cultivars. The infection potential values for the virus (MPN: infectious unit/gram of soil) are shown for each band in the four zones. Root disease severity observed in September is represented by 'R.S.'. Where there is no information, this is represented by a forward slash (/).

2. Materials and methods

To perform 454 sequencing reactions, three cultivars grown in the same field were chosen to create an amplicon library of cDNA of the target viral genes (*p14*, *p25* and *p26*) by reverse transcription polymerase chain reaction (RT-PCR). Viral genes producing proteins that had interaction with the plant were chosen. We obtained a total of nine amplicons. The amplicon library was sent to DNA Vision, where they were amplified in an emulsion drop PCR (emPCR). After the amplification stage, the DNA amplicons and the amplification bead were centrifuged onto a micro-titer plate, where they were ready to be sequenced. Between 6000 and 7000 sequences were obtained per amplicon.

2.1 Plant samples (from fields)

The site selected for this study in 2010 was a homogeneous field in Yèvres-la-Ville in Loiret, France (Figure 3.3). Each plot was subdivided into 12 zones corresponding to three varietal bands, and each band was subdivided into four zones. The sowing was done in 24 rows by 60 m per varietal band, and within each band four rows by 10 m were identified using the GPS. The different assays were 60 m long and 40 m wide. The beet cultivars used included one *Rz1rz1Rz2rz2* cultivar, one *Rz1rz1* cultivar with weak resistance and one *Rz1rz1* cultivar. The level of partial resistance was assessed by ITB. The tests were carried out on single sugar beet roots in May (12 samples per field), at the beginning of the growing season and in September (12 more samples per field) at the end of the season. The presence of RNA2, RNA3 and RNA5 of BNYVV, BSBV, BVQ, BBSV and *Polymyxa betae* was detected by duplex and multiplex RT-PCR, and the infectious potential of the soil was evaluated (Meunier *et al.*, 2003; Tuitert, 1990). The RNA2 (*p14* and *p15*), RNA3 (*p25*) and RNA5 (*p26*) sequences of BNYVV were obtained by direct sequencing. For the deep-sequencing, three cultivars (*Rz1rz1* cultivars with weak resistance ['S'], *Rz1rz1* cultivars with good resistance ['T'] and *Rz2rz2Rz1rz1* cultivars ['R']) were analyzed. The Yèvres-la-Ville field was homogeneously infected with the P-pathotype of BNYVV with the SYHG tetrad and the fifth RNA. Root disease severity assessed in September was 6% on 'S', 2% on 'R' and 22% on 'T'. 'S' showed better partial resistance than 'T'. The previous crop had been onions and, before that, winter barley. The last sugar beet crop grown on the field had been in 2007. The results obtained by field pattern and soils dried, using

the susceptible cultivar Cadyx (*rz1rz1rz2rz2*) in bioassay are shown in Figure 3.3.

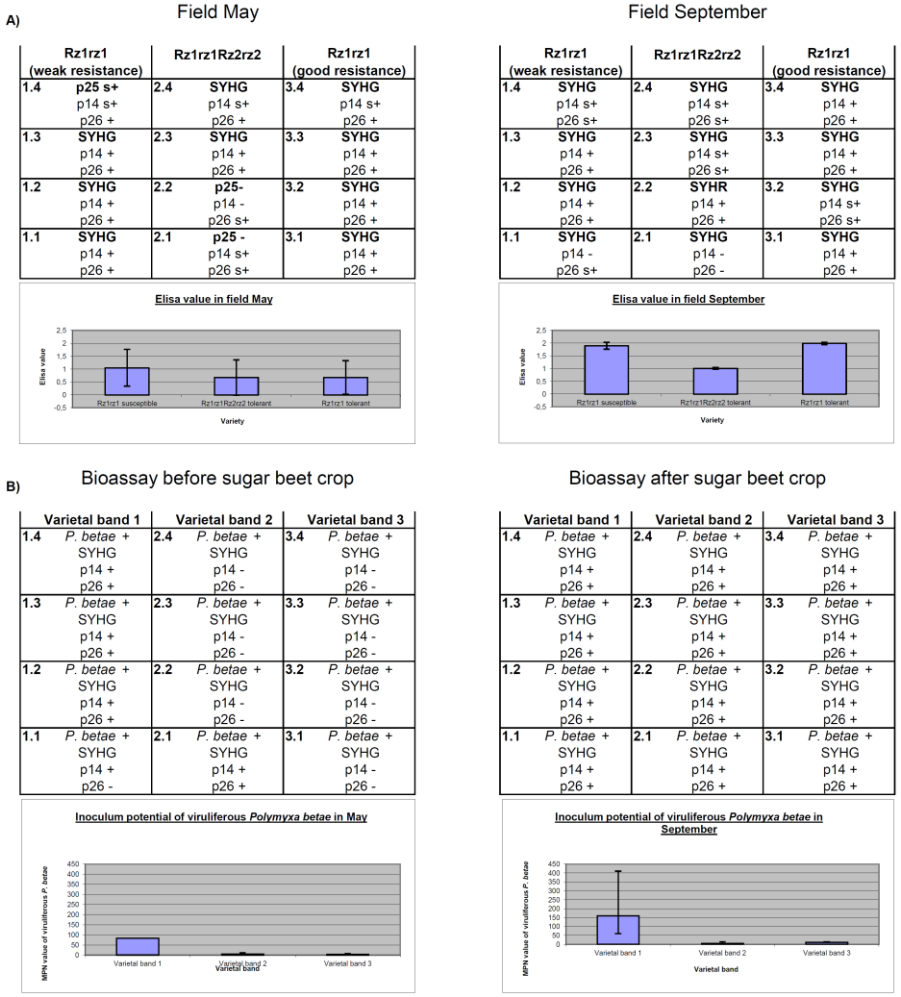

Fig. 3.3. Representation of a field assay (60 m long x 40 m wide) subdivided into three varietal bands and four zones. The experimental field at Yèvres-la-Ville (2010) was uniformly infested by BNYVV at start of the growing season. BNYVV was detected in each of the 12 subplots in a individual sugar beet, by direct RT-PCR detection as well as bio-assays based on soil samples. Apart from the presence of BNYVV, rhizomania-associated pomoviruses (BSBV and BVQ) were also detected, almost as uniformly as BNYVV. The presence of BSBMV, BBSV or sugar beet nematodes was checked and excluded. A) Results for BNYVV obtained by RT-PCR and sequencing of a individual sugar beet from the field at Yèvres-la-Ville in May and September 2010; B) Results for BNYVV and *Polymyxa betae* obtained from the bioassay by soils (sampled before and after sugar beet was cultivated) and dried using the Cadyx cultivar. The first number in each field zone relates to a cultivar: 1 = *Rz1rz1* cultivar with weak resistance (due to genetic erosion of the minor genes); 2 = *Rz1rz1Rz2rz2* cultivar; and 3 = *Rz1rz1* cultivars with good resistance. The second number in each zone relates to the four zones in each varietal band in the field. For each subplot, the sequence of BNYVV *p25*, *p14* and *p15* was obtained (Accession numbers: ERP002169) in both May and September, as well as from direct sequences from field sugar beet and bioassays. The code s+ represents a slightly detected gene. This approach confirmed the uniformity of the BNYVV detected, with SYHG and P-pathotypes for every subplot sampled in May, whatever the sugar beet cultivar background. The observed situation was fairly

similar for both sugar beet and soil samples collected at the end of the growing season in September, but in one plot as SYHR tetrad did appear in one of the four *Rz1Rz2* subplots.This mutation was not evidenced in the bioassays based on soils and using a susceptible cultivar (r*z1rz1rz2rz2*). This finding raised the question of whether the resistance background of the different sugar beet cultivars, leading to the set up of a deep sequencing strategy to evidence some possible changes within the BNYVV at the field level, was effective during a single crop.

2.2 Nucleic acid extract

The assay samples in 2010 were lyophilized and carefully ground. Total RNA was extracted from a homogenized single sugar beet root using the RNeasy plant mini kit (Qiagen, Germany). For the bio-assay, nucleic acid was also extracted using a modified procedure described by Jupin *et al.* (1990) and a polysome grinding buffer (Jackson and Larkins, 1976), using the Fastprep® system (Qbiogene, MP Biomedicals, California, USA).

2.3 Fusion primers

Nine pairs of primers had been designed for the RT-PCR, allowing for the amplification of the cDNA amplicon library and for deep-sequencing (Table 3.1). There were three pairs of primers per cultivar. These pairs corresponded to the targeted zone (assessing the region as highly variable) on *p25*, *p14* and *p26*. The primers had been designed in a conserved region around these genes to create the cDNA amplicon library. The melting temperatures were 59 °C, 66 °C and 66 °C for *p14*, *p25* and *p26* respectively.

2.4 Reverse transcription and cDNA amplicon library

For broad-spectrum detection, cDNA synthesis was performed in two stages. First, 9.5 µl of reaction mix containing 0.5 µl of primer reverse, 9 µl of diethylpyrocarbonate (DEPC) treated water and 1 µl of RNA were incubated at 65 °C for 10 min. Second, a reaction mix composed of 4 µl of M-MLV RT buffer (Promega Corporation, Madison, WI, USA), 2 µl of dNTP (20 nmol), 0.25 µl of M-MLV Reverse Transcriptase (200 U/µl) (Promega Corporation, Madison, WI, USA) and 3.25 µl of DEPC treated water was added to the first reaction mix. The total mix was incubated at 42 °C for 60 min.

2.5 PCR amplification and deep-sequencing

For PCR detection, the cycling times and temperature were 94 °C for 2 min (1 cycle), 94 °C for 30 s, melting temperature (°C) for 30 s, 72 °C for 30 s (35 cycles) and 72 °C for 7 min (1 cycle), being conserved finally at 4 °C. The reaction component for each sample detected was 26.25 µl sterile water, 5 µl $MgCl_2$ (25 mM), 10 µl Green Gotaq® Flexi buffer (Promega Corporation, Madison, WI, USA), 1.5 µl dNTPs (20 nmol), 1 µl each fusion primer (20 pmol), 0.25 µl Pfu DNA polymerase High Fidelity (5 U/ µl) (Promega) and 5 µl cDNA. For the initial identification of the tetrad, the amplicon band had been extracted from the electophoresis gel and purified before direct

Table 3.1. Fusion primers used for deep-sequencing.

Primer name	Sequence (5' → 3')				nt position (reference gene)	Product size (bp)	Beet cultivar
	Adaptor	Key	MID	Primer			
Bp14fSf	CGTATCGCCTCCCTCGCGCCA	TCAG	CGTGTCTCTA	TGCTACCAAAGAGCACGATG	RNA-2: 3851 to 4512 (NC_003515.1)	732	
Bp14fSr	CTATGCGCCTTGCCAGCCCGC	TCAG	CTCGCGTGTC	ACCACAACAACCTTCGGAAC			
Bp25fSf	CGTATCGCCTCCCTCGCGCCA	TCAG	CGTGTCTCTA	CCGTCATGGGTGATATATTAGGCGC	RNA-3: 440 to 1091 (NC_003516.1)	722	Rz1 (weak resistance)
Bp25fSr	CTATGCGCCTTGCCAGCCCGC	TCAG	CTCGCGTGTC	TCAACACCGTCAGGGGCTTGA			
Bp26fSf	CGTATCGCCTCCCTCGCGCCA	TCAG	CGTGTCTCTA	ATGGCATATAGCGACGATAATCACC	RNA-5: 455 to 1143 (NC_003513.1)	771	
Bp26fSr	CTATGCGCCTTGCCAGCCCGC	TCAG	CTCGCGTGTC	GCGGGTAGTTTAACGTCTTCGGAC			
Bp14fRz1f	CGTATCGCCTCCCTCGCGCCA	TCAG	TAGTATCAGC	TGCTACCAAAGAGCACGATG	RNA-2: 3851 to 4512 (NC_003515.1)	732	
Bp14fRz1r	CTATGCGCCTTGCCAGCCCGC	TCAG	TCTCTATGCG	ACCACAACAACCTTCGGAAC			
Bp25fRz1f	CGTATCGCCTCCCTCGCGCCA	TCAG	TAGTATCAGC	CCGTCATGGGTGATATATTAGGCGC	RNA-3: 440 to 1091 (NC_003516.1)	722	Rz1 (good resistance)
Bp25fRz1r	CTATGCGCCTTGCCAGCCCGC	TCAG	TCTCTATGCG	TCAACACCGTCAGGGGCTTGA			
Bp26fRz1f	CGTATCGCCTCCCTCGCGCCA	TCAG	TAGTATCAGC	ATGGCATATAGCGACGATAATCACC	RNA-5: 455 to 1143 (NC_003513.1)	771	
Bp26fRz1r	CTATGCGCCTTGCCAGCCCGC	TCAG	TCTCTATGCG	GCGGGTAGTTTAACGTCTTCGGAC			
Bp14fRz12f	CGTATCGCCTCCCTCGCGCCA	TCAG	TGATACGTCT	TGCTACCAAAGAGCACGATG	RNA-2: 3851 to 4512 (NC_003515.1)	732	
Bp14fRz12r	CTATGCGCCTTGCCAGCCCGC	TCAG	TACTGAGCTA	ACCACAACAACCTTCGGAAC			
Bp25fRz12f	CGTATCGCCTCCCTCGCGCCA	TCAG	TGATACGTCT	CCGTCATGGGTGATATATTAGGCGC	RNA-3: 440 to 1091 (NC_003516.1)	722	Rz1 + Rz2
Bp25fRz12r	CTATGCGCCTTGCCAGCCCGC	TCAG	TACTGAGCTA	TCAACACCGTCAGGGGCTTGA			
Bp26fRz12f	CGTATCGCCTCCCTCGCGCCA	TCAG	TGATACGTCT	ATGGCATATAGCGACGATAATCACC	RNA-5: 455 to 1143 (NC_003513.1)	771	
Bp26fRz12r	CTATGCGCCTTGCCAGCCCGC	TCAG	TACTGAGCTA	GCGGGTAGTTTAACGTCTTCGGAC			

sequencing. The sequences obtained were initially checked via the NCBI online database software (http://www.ncbi.nlm.nih.gov). The amplicon libraries used for deep-sequencing were purified through MSB Spin PCRapace (Invitek, Berlin) before sending for analysis by DNA Vision (Belgium). They applied Roche's 454 pyrosequencing on amplicons. The sensivity of variant detection is directly linked to the fold-coverage of the region (amplicon) where the variant appears. Therefore, a depth of coverage of 1000 times was chosen to be a good representation of the population of the variants present in each sample. DNA Vision provided raw sequences corresponding to clonal amplification.

2.7 Bioinformatic analysis

Raw data were analyzed using NGS Galaxy tools (Giardine *et al.*, 2005; Goecks *et al*., 2010; Blankenberg *et al*., 2010). Fastq files were first groomed. The quality of the sequences was then checked with the fastq quality trimmer by sliding window (score = 30). A mapping between the BNYVV reference genome (D84411.1 for *p14*, DQ682454 for *p25* and U78293.1 for *p26*) and the obtained sequences was done using BWA software (Li and Durbin, 2010). The map obtained was then read with the IGV software (The Broad Institute; Robinson *et al.*, 2011). Complementary analyses were made using Geneious Pro 5.6.6 software in order to make alignments and phylogeny trees. The FEL evolutionary model software (http://www.datamonkey.org) was used to estimate the ratio ω (d*N*/d*S*) by analyzing which codon sites in the alignment were subject to positive or negative selection (Poon *et al*., 2009). To get an idea of the location of the substituted aa and the conformation, a predicted secondary structure was made using I-Tasser (Roy *et al.,* 2010; Zhang, 2007).

3. Results

3.1 BNYVV field distribution

In the samples from the assays in the Pithiviers area, two tetrads were predominant: AYHR associated with B-pathotype BNYVV and SYHG associated with P-pathotype BNYVV (Galein *et al.*, 2011, 2013). Preliminary trials carried out in the field investigated by deep-sequencing showed homogeneous infection of the BNYVV virus by the SYHG tetrad. The field was in a rhizomania-infested zone, south of Paris, where single U/C substitution in the *p25* gene linked with a resistance-breaking event is very rare but disease problems are more often linked with the presence of the additional RNA5. All the results for the field distribution of BNYVV and *Polymyxa betae* are presented in Figure 3.3. Other rhizomania soil-borne viruses were also detected. For each plot in the Yèvres-la-Ville field, sequences of BNYVV *p25*, *p14* and *p15* were obtained in May and September, as well as from direct sequences from field sugar beet plants and bio-assays. This approach confirmed the uniformity of the BNYVV detected, with SYHG and P-pathotypes for every plot sampled in May, whatever the sugar beet cultivar background. The observed situation was

fairly similar for both sugar beet and soil samples collected at the end of the growing season in September, except for one plot where an SYHR tetrad appeared in one of the four *Rz1Rz2* plots. This mutation was not evident in the bio-assays based on soils when a susceptible cultivar (*rz1rz1rz2rz2*) was used. This result raised the question of the impact of the resistance background on viral micro-evolution in a single crop season leading us to the deep-sequencing strategy.

3.2 Sequence distribution

The distribution of all sequences from deep-sequencing (accession numbers: ERP002169) was obtained for each fusion primer when comparing the number of sequences obtained for each sequence length. The analysis program (Geneious Pro 5.6.6) was used to obtain the number of sequences for each fusion primer from deep-sequencing. According to the BNYVV gene analyzed, we obtained between 696 and 3,947 sequences per gene. Also obtained for each case were the maximum (559 bp) and minimum (5 bp) length, the minimum (between 280-320 bp) and maximum (between 500-559 bp) length extraction, the maximum length after end cutting and sequence treatment (between 354-459 bp), the number of sequences for tree construction (between 514 and 1,616 for *p14*; between 1,676 and 2,274 for *p25*; and between 1,219 and 2,357 for *p26*), the percentage pairwise (%) (between 98 and 99.4%) and the percentage identical site (between 6.1 and 36.4 for *p14*; between 7.2 and 9.7 for *p25*; between 4 and 14.3 for *p26*).

3.3 Sequence depth/mutations

The depth coverage of the entire gene was investigated (*p25*, *p26*, end of *p15* and *p14*). Good coverage was obtained for all major mutation positions. For *p25*, a minimum coverage of 31 sequences was observed only in the nucleic acid position from 304 to 306. For *p26*, there was a minimum coverage of 639 sequences at position 360 in the nucleic acid of the *p26* gene within the *Rz1rz1* cultivar with weak resistance. With regard to the end of *p15*, there was also a minimum coverage of 359 sequences at position 382 in the *Rz1rz1* cultivar with good resistance. For *p14*, the minimum coverage was 246 sequences at position 239 within the *Rz1rz1* cultivar with good resistance. With regard to reverse sequences, the major mutation positions were far enough from the sequence ends to be considered.

3.4 Changes in the nucleotides/amino acids of BNYVV depending on the sugar beet cultivars

The distribution of all sequences was obtained for each fusion primer (targeting *p14* and *p15* C-terminus, *p25* and *p26* of BNYVV) and compared with reference sequences for *p25*, *p26*, *p15* C-terminus and *p14*, respectively (accession number DQ682454 from Pithiviers for the P-pathotype *p25*, U78293.1 from Europe for *p26* and D84411.1 from Japan for *p14* and *p15*). For each gene (*p14*, *p15*, *p25* and *p26*) from each cultivar, the mutation percentage (%) was obtained as a function of the nucleic acid

Table 3.2. Mutation proportion for each mutated amino acid of the p15, p14, p25 and p26 protein in the various beet cultivars (*Rz1rz1* cultivars with weak resistance, *Rz1rz1 Rz1* cultivars with good resistance and *Rz1rz1Rz2rz2* cultivars).

BNYVV Gene	Cultivar	Amino acid position and proportion of each amino acid											
p15	Rz1rz1 (weak)	83 100% A	95 100% R										
	Rz1rz1 (good)	34% G & 66% A	100% C										
	Rz1rz1Rz2rz2	3% G, 9% V & 89% A	100% C										
p14	Rz1rz1 (weak)	73 1% Q & 99% R	77 1% Y, 2% S, 2% F, 2% W & 93% C	78 1% E, 2% Q & 97% K	79 3% E & 97% K	80 2% K, 4% P & 94% Q	81 1% S, 14% I, 14% K & 71% N						
	Rz1rz1 (good)	1% Q & 99% R	3% S, 3% F, 3% W & 91% C	3% Q & 97% K	4% E & 96% K	3% K, 5% P & 92% Q	15% I, 15% K & 70% N						
	Rz1rz1Rz2rz2	4% Q & 96% R	1% S, 1% F, 1% W & 97% C	1% Q & 99% K	1% E & 99% K	1% K, 2% P & 97% Q	4% I, 4% K & 92% N						
p25	Rz1rz1 (weak)	25 72% G & 28% D	31 27% A & 73% T	32 1% N, 1% Y & 98% H	55 100% N	**67 (Tetrad)** 1% T, 1% P & 98% S	**70 (Tetrad)** 100% G	88 3% P & 97% R	132 3% R & 97% I	163 100% P	**209 (& in gene N)** 95% S & 5% I		
	Rz1rz1 (good)	99% G & 1% D	100% T	2% Y & 98% H	100% N	100% S	100% G	100% R	100% I	2% S & 98% P	1% N, 5% S & 94% I		
	Rz1rz1Rz2rz2	100% D	100% T	1% N & 99% H	9% S, 1% K & 90% H	100% S	100% R	100% R	100% I	100% P	100% I		
p26	Rz1rz1 (weak)	31 1% A & 99% V	77 100% D	92 100% F	117 3% R & 97% S	120 1% V & 99% D	124 1% F & 99% L	139 3% N, 3% F & 94% Y	140 3% W & 97% C	146 2% V & 98% E			
	Rz1rz1 (good)	100% V	47% V & 53% D	1% L, 1% V & 98% F	1% R & 99% S	1% G, 1% V & 98% D	2% F & 98% L	1% N, 1% F & 98% Y	1% W & 99% C	1% V & 99% E			
	Rz1rz1Rz2rz2	100% V	1% V & 99% D	100% F	5% R & 95% S	1% G & 99% D	2% F & 98% L	2% N, 2% F & 96% Y	2% W & 98% C	1% V, 1% A & 98% E			

Rem. The results obtained for the p15 protein corresponded only to the end of the *p15* gene because the forward primer targeting the *p14* gene was located at the end of *p15*. For the p14 protein, there were five mutations located one after the other. This corresponded to a region rich in lysine. There were high proportions of mutation in the p14 protein and the amino acid position with the highest proportion was 81. In the p25 protein, there were 10 positions between each cultivar. The highest proportions were observed at positions 25 and 31, followed by position 55. In p26, there were nine positions. The highest proportion was observed at position 77. The depth coverage for all the genes was investigated (*p25*, *p26*, *p15* C-terminus and *p14* gene). A gap coverage for the *p25* gene in the *Rz1rz1* cultivar with weak resistance was observed. This was due to the experiment and to the use of forward and reverse primers with no consensus. In the other cultivars, the minimum coverage was 31 sequences at the acid nucleic position 304 to 306. These sequence gaps were outside the major mutation position, so that the results were robust. For p26, overall there was a good coverage, with a minimum coverage of 639 sequences at position 360 in the nucleic acid of *p26* in the *Rz1rz1* cultivar with weak resistance. For the end of *p15*, there was also good coverage, represented by a plateau with a minimum coverage of 359 sequences at the acid nucleic position 382 in the *Rz1rz1* cultivar with good resistance. For *p14*, the minimum coverage was 246 sequences at position 239 in the *Rz1rz1* cultivar with good resistance. This is problematic because this lower coverage appeared near the major mutation position. When the different reverse sequences were investigated, the major mutation positions were observed at the acid nucleic positions 242 and 243 in *p14* and in several cases were far from the end of the reverse sequences, which was the case for several sequences where this major mutation position was 180 bp from the end of the sequences. This allowed this mutation, nevertheless, to be taken into account.

position. The mutations in major aa positions are shown for each gene in Table 3.2, apart from silent mutations. For each of the genes targeted, mutations specific to particular sugar beet cultivars were observed.

For *p15* C-terminus, two major mutation positions, causing an aa change and thus functional changes, were observed. These mutations differed depending on the cultivar. The position showing the highest rate of mutation was aa 95. With regard to p14 protein, there were six major changes in aa positions. These changes also differed among the beet cultivars. Five of the six positions, located one after the other (aa 77 to 81), were positioned in a lysine rich region and were subject to changes. The highest mutation rate was observed for position 81. More aa variation was noted in *Rz1* cultivars with weak resistance. For the p25 protein, 10 major changes for the aa were observed. The mutation at position 626 in the nucleic acid sequence (aa 209 in p25) was therefore present in the gene N. The highest mutation rates were observed at positions 25, 31, 55, 70 and 209. The NLS and NES were not affected by aa mutation. An aa mutation (position 132) for the *Rz1* cultivars with weak resistance, however, was observed in the transcription activation domain. Moreover, in the tetrad plus zinc finger, only the tetrad was modified. For the p26 protein, between each cultivar there were nine functional changes in the aa. The highest mutation rate was observed at position 77.

3.5 Mutation effects on the amino acid sequence

In the p25 protein, there was a negative charge loss at aa position 25. At position 31, there was a replacement from a non-charged polar aa to a non-polar aa. At positions 32 and 55, there were also functional changes. At position 67, there was no change in the charge. At position 70 in the tetrad, there was an aa replacement from a non-polar aa to a positively charged aa. Positions 88, 132, 163 and 209 also showed variation in charge and function. The mutation at position 209 in the aa of p25 was also present in the aa of gene N. This position mutation could also affect the conformation and function of gene N.

In the p26 protein, there was a change at position 31 with a majority of valine at this position. At aa position 77 (the highest positive selection pressure with the highest aa change in proportion), there was a replacement from a negative charged aa to non-polar and non-reaction aa.

With regard to the p15 protein C-terminus, a replacement of a non-polar aa by another non-polar aa in position 83 was observed. At position 95, a replacement of a positive charged aa by a polar aa enabled a disulfide bond to be established. This replacement was observed in the *Rz1rz1* cultivars with weak resistance and *Rz1rz1Rz2rz2* cultivars.

In the p14 protein, the major mutation occurred at aa positions 73, 77, 78, 79, 80 and 81. A major replacement of a non-charged polar aa by an aa with a positive charge occurred at position 73. Only a few changes were

observed at position 74 in the p14 protein. Only 1% of the sequences within the *Rz1rz1* cultivars with weak resistance and *Rz1rz1* cultivars with good resistance exhibited the loss of a positive charged aa for a polar aa. For aa position 77, the replacement of a non-charged polar aa by an aromatic aa was observed in all cultivars. This phenomenon was more frequent in the *Rz1rz1* cultivars with good resistance. At position 78, up to 3% of the positive charge for a polar aa was lost within the *Rz1rz1* cultivars with good resistance. At position 79, a positive charge was transformed into a negative charge. Position 80 showed a mutation of 1-8% depending on the cultivar. Two nucleic acid mutations for this aa were observed. The mutation in the nucleic acid position 238 led to an aa change from a polar to a positively charged aa. This mutation therefore meant the gain of a positive charged lysine in this lysine-rich region. The nucleic acid position 239, however, allowed a replacement of an aa polar by another polar aa. No important change in function was induced here. More significant mutations occured at the two nucleic acid positions 242 and 243 encoding for aa 81. Mutation rates of 14-15% were observed at these nucleic acid positions (30% in total for the aa) for the *Rz1* cultivars, but for the *Rz1Rz2* cultivar the rate was only 4%. The position 242 replaced a polar aa by a non-polar one, but the position 243 resulted again in a lysine gain in this lysine-rich region. Mutation at position 83 in the protein also resulted in a 1% gain in lysine within the *Rz1rz1* cultivars with good resistance, with a change from a polar to a positively charged aa.

3.6 Errors rate

The sequences obtained in this study were analyzed using Geneious Pro 5.6.6 software and NGS Galaxy tools, and cleaned and formatted to discard any dubious sequences. An arbitrary cut-off threshold of 1% was also set as a minimum, as proposed in earlier studies (Kircher and Kelso, 2010; Larsen *et al*., 2012). The sequence length of each amplicon sent for deep-sequencing was about 750 bp, but ultimately the maximum length of each sequence, after 454 deep-sequencing, end cutting and sequence treatment, was about 400 bp.

3.7 Selection pressure analysis

The percentages of pairwise identity and of identical sites were also obtained. In general, for *p14*, *p25* and *p26* in each cultivar, the matrix identity between each sequence was between 94% and 100%. The different selection pressures (ω value) for each type of cultivar were also analyzed for *p14*, *p25* and *p26* in each aa position (data not shown). When several sequences were identical at the nucleotide level, only one sequence was retained. As a result, the analysis of the selective pressure of BNYVV was performed using between 129 and 495 sequences for *p14*, between 445 and 500 sequences for *p25* and between 382 and 500 sequences for *p26*. The three ORFs showed very different evolutionary patterns, even within the different cultivars. Knowing that we were in a field containing mainly the P-pathotype (tetrad SYHG), a positive selection pressure was not really

observed in the tetrad. The *p14* gene, however, was the most constrained one (more aa under high positive selection pressure).

4. Discussion

This study showed that the virus micro-evolves, in a single sugar beet season, depending on the cultivars grown in the field. For a single sugar beet root in a homogeneous field, we demonstrated the *p14*, *p25* and *p26* variability in P-pathotype BNYVV populations where the resistance background differed. The selection pressure among hybrids carrying *Rz1* and/or *Rz2* resistance with different levels of resistance clearly showed different levels of mutation frequency in the virus genome.

4.1 Sugar beet genotype influences the viral population

Deep-sequencing showed that there were different variants for the same pathotype of BNYVV (P-pathotype here) from the same field, depending on the cultivar grown (*Rz1* cultivar with weak resistance, *Rz1* cultivar with good resistance and *Rz1Rz2* cultivar). For the three genes analyzed (*p14*, *p25* and *p26*), major changes in the viral genome sequence and the aa of the corresponding protein were observed. These results indicate that the genetic background of the sugar beet host can shape the viral population during a single growing season. Similar results have been obtained for human viruses (Wang *et al.*, 2007a; Bull *et al.*, 2011). The results clearly raise the question of the significiance of such mutation at the level of plant-virus interaction. These mutations seem to indicate that the interaction of the virus with the plant need to be considered in a much broader context than as a simple interaction only with the p25 protein.

4.2 Mutation effects on the amino acid sequence

Mutations occurred in the basic region as well as in the tetrad of the p25 protein. With regard to the known functional domain of p25, the NLS (positive pattern of $_{57}$**KR**IRF**R**$_{62}$), NES (valine-rich non-polar pattern of $_{169}$**V**YM**V**CL**V**NT**V**N$_{178}$), Zn finger and transcription activation domain remained mostly unchanged (Hleibieh *et al.*, 2007). The major functional change in the protein should affect protein conformation and protein activity.

The results of the predicted secondary structure for the p25 protein showing a major functional change for the aa at position 25 allowed a change from an aa with a negative charge to a non-reaction aa. The basic region of p25 increased with this positivity. The basic region (positively charged) was important because it was probably the region linked with DNA (negatively charged). The basic region increased the accessibility to DNA and therefore allowed interaction and change in DNA. The mutation at position 70 in the tetrad could affect the virulence of the virus.

In the p26 protein, the change at position 31 was probably because it was a French population with mostly valine at this position. The European

reference (accession number U78293.1), however, showed an alanine at this position, whereas other countries showed a valine. The aa remained non-polar.

The p26 protein acted in a synergistic manner with p25 and seemed to be similar in the functional domain present in the protein (Tamada *et al.*, 1996; Link *et al.*, 2005). In this study, the $Fx_3FRGPGNx_2L$ sequence was found at the end of the transcription activation domain of p25 and in p26 (Hleibieh *et al.,* 2007). Nevertheless, fewer functional mutations showing high substitution rates were found in p26 than in p25. In addition, the mutations seemed different. A strongly positively charge was also found with a pattern $_{61}$**H**M**K**I**K**TM**R**$_{68}$ (aa position 61 to 68) that could correspond to an NLS (nuclear localization signal). At the aa positions 175 to 185, a rich valine zone pattern was found ($_{175}$**V**DSIY**V**AC**V**AS$_{185}$) that could be linked to an NES (nuclear exportation signal). Link *et al.* (2005), however, identified the p26 transcription-activation domain within its first 55 aa using alanine scanning. These findings suggested that amino acids between positions 55 and 110 could act as a cytoplasmic-retention sequence.

With regard to the protein p15 C-terminus, there seemed to be no effect of the substitution in position 83 on this part of p15. At position 85, valine seemed to be characteristic of the French P-pathotype. Other strains such as Japanese, as well as Swiss A-pathotype (isoleucine), P-pathotype (isoleucine) and J-pathotype (leucine), can be discriminated thanks to position 103. Hleibieh *et al.* (2007) also showed that eight variable residues (in p26: positions 30, 69, 77, 103, 142, 146, 149, 174, 200 and 227-229) could discriminate between P-pathotype and J-pathotype in the p26 protein.

With regard to the major substitution in the p14 protein, position 34 enabled discrimination to be made between the European BNYVV pathotype (glutamate) and the J-pathotype (aspartate). No functional change was observed at this position. All mutations observed in the p14 protein appeared in the same area (positions 73, 77, 78, 79, 80 and 81). The mutation at aa position 73 was because the fact that the sequence came from Europe. This 'mutation area' was a lysine-rich, polar and positively charged region. This basic region could interact with DNA (negatively charged). The pattern of this sequence was $_{70}$**K**CCR**K**L**K**C**KK**QN**K**N**H**S**KH**$_{87}$. At position 81, the ω values range observed for the different cultivars was between 6,294 and 9,738, which is considered a very strong positive selection pressure. Position 81 in p14 showed the highest mutation rate in the *Rz1* cultivar and a lower mutation rate in the *Rz1Rz2* cultivar.

The pattern between aa 70 to 87 seemed very variable and could be the site of interaction with other proteins or DNA. We hypothesized that this pattern could play a role in suppressing the post-transcriptional gene silencing of the plant (PTGS). This part of p14 could suppress silencing by sequestering both the long dsRNA and double-stranded siRNA components of the silencing machinery. Fewer mutations were observed in the *Rz1Rz2* cultivars than in the others. This lower mutation level could explain the better

resistance of the *Rz1Rz2* cultivars because of the lower inhibition of PTGS. The predicted protein secondary structure (I-Tasser) showed that this pattern was situated between two ß sheets. The pattern was composed of a mixture of colloids, a few parts of ß sheet and different part of α helix. If this protein interacted with DNA, p14 should also have had an NLS and an NES to shuttle in the nucleus. During secondary structure analysis (I-Tasser) (Roy *et al.,* 2010; Zhang, 2007), a potential region for NES near the pattern $_{9}$**V**F**V**GR**V**$_{14}$ rich in valine was identified. The potential region for NLS could be situated near the pattern $_{94}$**RK**V**R**N$_{98}$ with positive aa.

A decreased ability to adapt themselves was observed in animal virus isolates that had experienced repeated genetic bottlenecks (Novella *et al.*, 2010). These differences in adaptability suggested differences in the rate at which beneficial mutations were produced, or the effects of beneficial mutations, or both. Some evolved, high-fitness mutants might be less robust than evolved, high-fitness wild type ones (robustness is the ability of a virus to avoid phenotypic changes in the face of mutation). Fitness losses were significantly greater in mutant-bottlenecked populations than in wild populations (Novella *et al.*, 2010). No repeated genetic bottleneck was observed for BNYVV. The virus seemed to be constant and conserved over time and space (Koenig and Lennefors, 2000). Thus, mutant BNYVV isolates could attain greater fitness than wild populations.

4.3 Selection pressure

Under negative or purifying selection, less 'fit' non-synonymous substitutions accumulate more slowly than synonymous substitutions, whereas under diversifying or positive selection the opposite prevails (Li *et al.*, 1985, Yang *et al*., 2000). The genetic variation found in sequences is a combination of independent substitutions in different lineages and substitutions inherited by related sequences from a common ancestor (Kosakovsky Pond *et al.*, 2004a, 2004b, 2007).

Comparing the A-, B- and P-pathotype of BNYVV, Schirmer *et al.* (2005) showed a high selection pressure for 39 sequences for the aa 68 of the p25 protein with ω values of 25.1. A high ω values was also found for the aa 198 of p25. According to a second model, 13 aa sites belonged to the ω = 4.7 category of positively selected codons, with a confidence of P > 90%, and one aa site (position 68) belonged to the ω = 54.7 category of positively selected codons, with a confidence of P > 99.9%. The p26 protein (28 sequences) showed mean ω values varying from 0.20 to 0.33, depending on the model used. The aa sites 9, 81 and 143 of p26 belonged to the positive selection category (P > 90%). Chiba *et al.* (2011) also showed that the values of d_{NS}/d_S (ω) in Italy differed greatly for BNYVV genes encoding for the p25 protein (ω = 1.500), p31 (0.500) and CP (0.333). The *p25* gene was subject to very strong positive selection. Chiba *et al.* (2011) suggested that the existence of the highest selective pressure on the *p25* gene could influence selection pressure on the other genes. Alternatively, strong selection pressure could be imposed at the same time on all BNYVV

proteins. Such strong positive selection pressure on the *p25* gene was associated with resistant cultivar cropping.

In this study, only the micro-evolution in the P-pathotype was investigated. We analyzed three sugar beet genetic backgrounds and their influence on the BNYVV P-pathotype. Other sequences were analyzed (129-145 depending on the cultivar for *p14*; 445-500 for *p25*; and 382-500 for *p26*) to obtain a representative ω value. There were fewer nucleic acid positions in *p14* under positive selection pressure than in *p25* and *p26*. Nevertheless, high positive selection pressure was observed. For the p14 protein, in the *Rz1* cultivar with weak resistance the aa 55 (2.079), 59 (1.099), 77 (1.095), 81 (6.294) were under very strong positive selection pressure. In the *Rz1* cultivar with good resistance, the aa 16 (3.974), 42 (3.558) and 81 (9.738) were under strong positive selection pressure, whereas for the *Rz1Rz2* cultivar, this pressure concerned the aa 56 (9.086), 88 (2.898) and 116 (3.465). The highest positive selection pressure was observed for the aa 81 in the p14 protein, with a ω value of 6.294 in the *Rz1* cultivar with weak resistance and of 9.738 in the *Rz1* cultivar with good resistance. The *Rz1* seemed to apply strong pressure on this aa. No values for this aa, however, were obtained for the double resistance cultivar. For the p25 protein, more aa were under positive selection pressure, but to a lesser extent than that observed for p14. In the *Rz1* cultivar with weak resistance, the aa 27 (1.025), 108 (1.062) and 160 (1.567) were under very strong positive selection pressure. In the *Rz1* cultivar with good resistance, this pressure concerned the aa 81 (1.124), 92 (1.192), 101 (1.056), 145 (2.590), 160 (1.717) and 176 (1.776). For the *Rz1Rz2* cultivars, the aa 55 (4.317), 115 (1.221), 123 (1.183) and 176 (1.219) were those under higher positive selection pressure. In p26, fewer aa were under positive selection pressure. For the *Rz1* cultivar with weak resistance, only five aa (61 [1.015], 142 [1.101], 153 [1.047], 170 [1.815] and 180 [1.509]) were under positive selection pressure, whereas for the *Rz1* cultivar with good resistance (aa 77 [11.625], which is the highest ω value observed), and the *Rz1Rz2* cultivar (aa 162 [1.139]), only one aa was under positive selection pressure.
Consequently, the p14 protein seemed to be under higher positive selection pressure than p25 and p26, but for less aa overall. The mutation advantage that gave a fitness advantage over the others appeared to differ, depending on the cultivar. Theses mutations seemed well controlled by the *Rz1rz1Rz2rz2* cultivar (few aa positions under selection pressure) and the selection position changed as a function of the cultivars.

4.4 Error rate

An arbitrary 1% cutoff is often used to distinguish background error from real variants (Kircher and Kelso, 2010; Larsen *et al.*, 2012, Schmitt *et al*., 2012). The study by Mild *et al.* (2011) demonstrated that ultra-deep pyrosequencing (UDPS) resulted in low experimental noise and high repeatability (for variants representing > 0.27% of the HIV virus population, whereas some variants representing 0.11-0.27% were detected in only one of the two UDPS run), which is relevant. The 1% cutoff was also applied in this study.

5. Conclusions

In this study, in the overall context, it was observed that the virus micro-evolved and changed a little in a single sugar beet season, depending on the different cultivars in the field. In a single sugar beet root and in a homogeneous field, we demonstrated *p14*, *p25* and *p26* variability from P-pathotype BNYVV populations among different resistance backgrounds. Thanks to NGS, the impact of different resistance backgrounds can now be evaluated at the root scale.

The beet genotype influenced the viral population. Similar results were observed for other pathosystems. Beyond the influence of the genotype of the host, however, the vector also constitutes a bottleneck that can influence the ability of the current move from one season to another. In their study, Bornemann and Varrelmann (2013) confirmed these observations in controlled conditions.

The observed diversity from one field to another was consistent if only the p25 tetrad was taken as an indicator. This raises the question of the origin of this diversity, in which beet genotype clearly played a significant part.

The diversity also raised the question of their impact on the virus-beet interaction. Although it is worth checking the hypothetical effects of these mutations, the results of this study suggested that the virus-beet interaction actively involved several viral genes.

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CHAPTER 4

Results analyses

4.1. Questions and hypotheses

Several questions emerged from the huge quantity of different results obtained from the field and the bio-assays between 2009 and 2012. For the different cultivars used, ELISA results, root severity per varietal band, AYHR and SYHG tetrad quantity, the presence or not of the RNA3 (1344 tetrads), RNA2 and RNA5 of BNYVV, the presence or not of BVQ, BSBV, nematodes and *Polymyxa*, and the soil component from the field were obtained. The infectious potential with the viral and vector MPN were obtained from the bio-assays. There was a lot of data but what is the main component, the parameter which explains the root severity and the yield loss in the field?
Theses different questions were: is the increase of the viral content linked to a specific tetrad? Is there an evolution of the BNYVV B-pathotype and P-pathotype content in the sugar roots from the field in a beet season? Is this increase linked to the root severity (which is our disease impact indicator), knowing that root severity is linked to an auxin perturbation? Is the root severity linked to a specific tetrad? Is the root severity linked to the virus MPN and/or vector MPN? Is root severity linked to the nematode cysts or juveniles? Is the tetrad found in the field sample linked to the infectious potential of the soil? Is the virus MPN linked to the vector MPN? Is it due to something else in the virus level which is not put in evidence with our indicators? Is it due to an external parameter as virus-nematode synergism or associated viruses? Is there an evolution of the infectious potential in the soil in a beet season? How the different varieties used behave in different soils contexts? Is there a varietal effect on the infectious potential during the beet season? Is there a varietal effect on the virus? In case of tetrad mixed infection in a single sugar root, what is the proportion of each tetrad in the sample? Is there a selection pressure of the different varieties used on the tetrad diversity or a natural inter-field diversity? Must we lengthen the rotation period in order to decrease the infectious potential? To answer to

these different questions, parameters correlations were made. The different results are presented below in this section.
The RT-mqPCR was also investigated to evaluate the presence of the BNYVV B-pathotype and P-pathotype. These results came from the varieties used in the ten field implemented in September 2010. There were 38 samples selected for the analyses by RT-mqPCR. In the different varieties, there were simple infection of each pathotype and also double infection of pathotype. This technology gave us also some answers to some questions. The RT-mqPCR results performance was compared to the ELISA values.

4.2. Is the increase of the viral content linked to a specific tetrad?

The figure 4.1 and 4.2 shows that the tetrad SYHG linked to the P-pathotype was also linked to higher ELISA values for the virus content in a single beet root. The ELISA values increased during the season when the tetrad SYHG was observed. But, the ELISA values decreased in the season when the tetrad AYHR was observed.

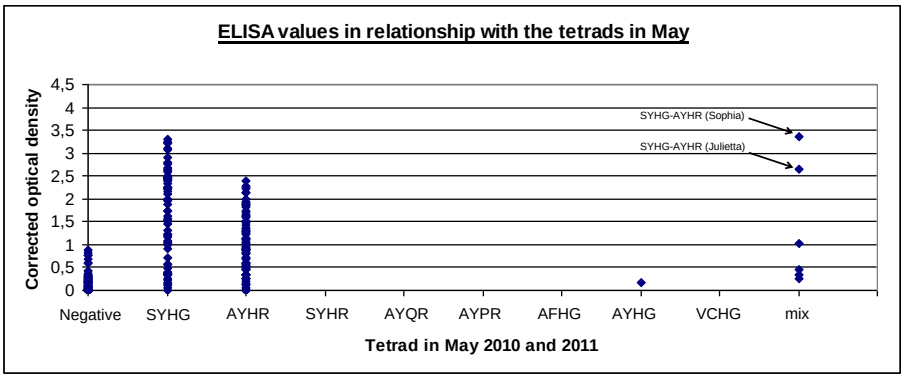


Fig. 4.1. Correlation relation between the ELISA values (corrected OD) and the tetrads of 240 samples from May 2010 and 2011.

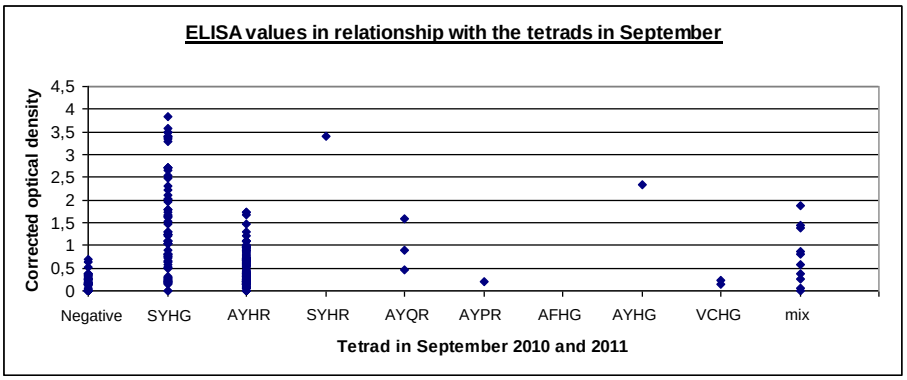


Fig. 4.2. Correlation relation between the ELISA values (corrected OD) and the tetrads of 240 samples from September 2010 and 2011.

4.3. Is the root severity linked to a specific tetrad?

The figure 4.3 shows that the tetrad the more present in the fields for 2010 and 2011 was AYHR linked to the B-pathotype. The overall results from these two years were considered independently of the variety used. Moreover, this AYHR tetrad was linked to the highest root severity. The value range was comprised between 0 and 55% of root severity while the value range for the SYHG tetrad was comprised between 0 and 35%.

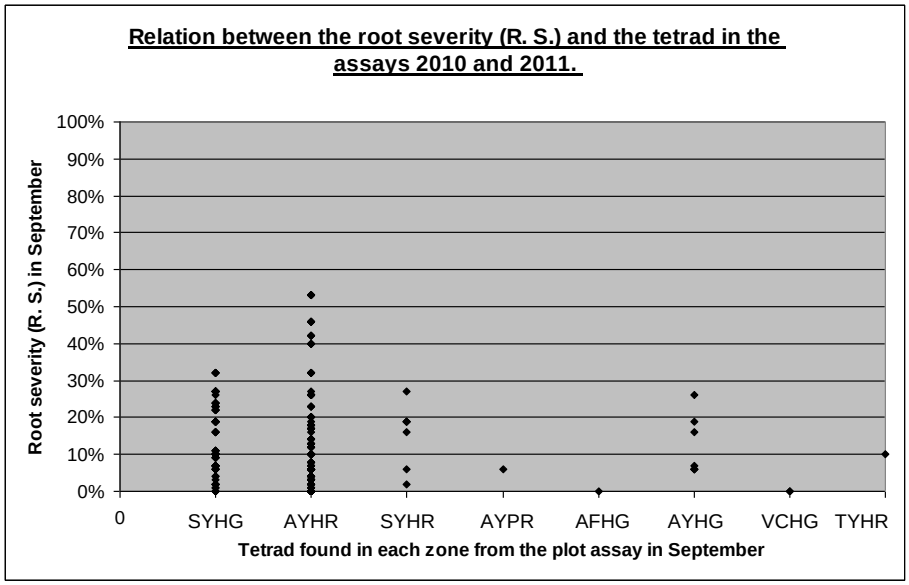


Fig. 4.3. Relation between the root severity and the tetrad observed in the assays in the field in 2010 and 2011.

4.4. Is this increase of viral content linked to the root severity?

According to the results obtained in 2010, we didn't observe a correlation between the root severity and the ELISA values. Indeed, for different root severity we had a different level of ELISA values. However, the figure 4.4 shows that the highest ELISA values were linked to the SYHG tetrad (P-pathotype) in simple infection independently of the root severity.

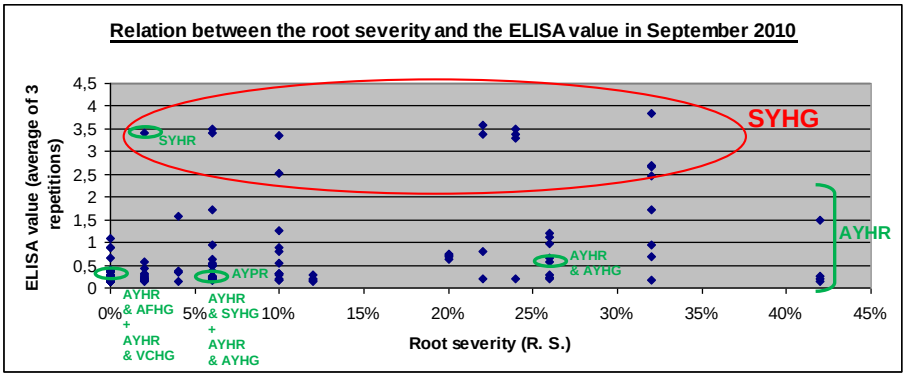


Fig. 4.4. Relation between the root severity and the ELISA values (corrected OD) in September 2010 (one single sugar beet root).

The different RT-mqPCR results can't be explained directly by the root severity observed in the field. There was no strict correlation between the two parameters (data not shown).

4.5. Is the root severity linked to the virus MPN and/or vector MPN?

There were no relation between the root severity and the logarithm of the MPN. The logarithmic scale in base 10 is used to represent phenomena that may vary for example 10^{-10} to 10^{10}. It allows to amplify the variations of values near to zero and to make less important the variation for the biggest number, putting in evidence rather the relative variations. The root severity was not really linked to the vector MPN. The root severity explained only for 16,19% the virus MPN. Indeed, for a multiplication factor of 1.5 or 3 in the virus MPN, we observed the same root severity. But, a multiplication factor of minimum 2 in the vector MPN was requested to have a root severity. This corresponded to a minimum of 100 infectious unit of vector per gram of soil.

4.6. Is root severity linked to the nematode cysts or juveniles?

The root severity in the varieties resistant against to BNYVV and nematode was explained for 13,9% by the multiplication rate during the beet season of the nematode cysts (Figure 4.5). However, the juvenile present in the beginning of the culture and at the end of the season did not influence the rhizomania root severity observed in the field in September (data not shown).

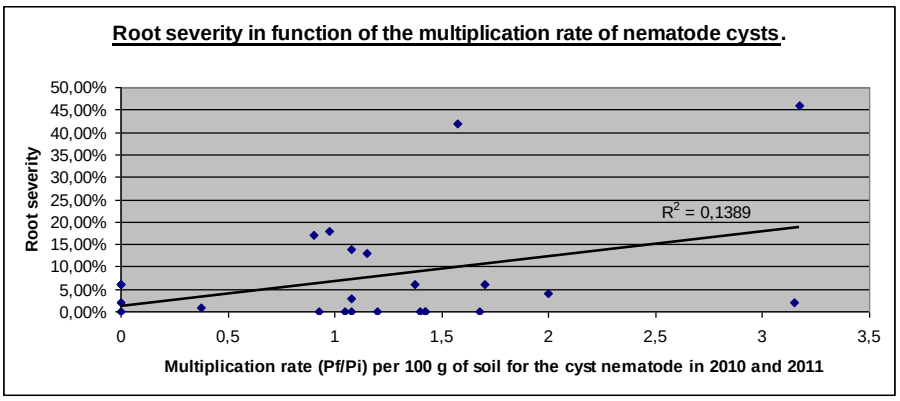


Fig. 4.5. The root severity as a function of multiplication rate (Pf/Pi) per 100 g of soil for the cyst nematode for 96 samples from 2010 and 2011.

4.7. Is the virus MPN linked to the vector MPN?

The scatterplots are useful to verify the correlation existence of a relation between two quantities (possible cause-effect between two phenomena). They bring not always the formal proof that a variable imply the other, but show if a relation between the both exists and the strength of this one. We can observe here, in the figure 4.6, a regression line very slightly positive and linear. When the vector MPN increased, the virus MPN increased also but to a lesser extent.

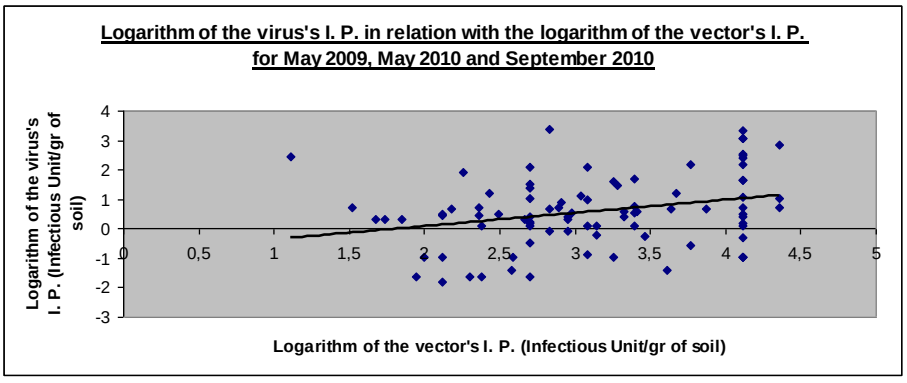


Fig. 4.6. Logarithm of the virus MPN (infectious potential) in relation with the logarithm of the vector MPN (infectious potential) for May 2009, May 2010 and September 2010.

The linear correlation coefficient which defines the intensity and the direction of a linear relation between two quantitative variables is defined by r (between -1 and 1) and was equal to 0,289. The coefficient of determination is the r^2 of the linear correlation coefficient and was equal to 0,084. It evolve from 0 (no linear relation) to 1 (relation perfectly linear, positive or negative). The t test (Student) of comparison of the average of two observation groups showed a t-value of 2,804 and a p-value of 0,006. The degree of freedom, which is the sum of each variable in each group minus two, was equal to 86.

T shows that it doesn't really exist a relation between the dependent variable and the independent variable. The average for the virus MPN was 0,5 and 2,3 for the vector.

4.8. Is there an evolution of the infectious potential in the soil in a beet season?

Yes! The vector MPN increased strongly and the viral MPN increased also to a lesser extent. This is shown in the table 4.1 with the multiplication rate between the beginning of the culture and the end ot the season.

Table 4.1. Varietal effect on the virus (in 2010 and 2011) and comparison between "before the beginning of the beet culture" and "in the period of beet harvest" per varietal band.

Varietal effect on the virus in 2010 and 2011 and comparison between "before culture" and "after culture"											
Year	Variety	IP May (variety band (MPN virus/MPN vector)	IP Sept. (variety band (MPN virus/MPN vector)	Multiplication rate in the band	Root gravity in September	Tetrad in biossay in May	Tetrad in biossay in September	Tetrad in assay in May	Tetrad in assay in September	Average ELISA values in May	Average ELISA values in September
2010	AL Ludwinia	3,6/940	3,2/310	*0,88/*0,33	10%	SYHG	SYHG	SYHG	SYHG	0,723	1,858
	BL Ludwinia	4,6/150	5,2/13000	*1,13/*86,67	2%	SYHG	SYHG	SYHG	SYHG-SYHR	0,673	1,008
	GL Ludwinia	2/890	1,2/13000	*0,6/*14,61	10%	AYHR	AYHR	Undeterminated	AYHR	0,052	0,219
	Al Ludwinia	120/1200	360/13000	*3/*10,83	10%	AYHR	AYHR	AYHR	AYHR	0,682	0,629
	Bl Ludwinia	1,2/500	46/13000	*38,33/*26	6%	AYHR	AYHR	/	SYHG-AYHR	0,241	0,314
	Cl Ludwinia	0,32/500	2,5/2100	*7,81/*4,2	0%	AYHR	AYHR	/	VCHG-AYHR	0,076	0,191
	AL Python	5,2/33	40/1800	*7,69/*54,54	32%	SYHG	SYHG	SYHG	SYHG	1,085	2,914
	BL Python	2,6/890	12/13000	*4,6/*14,61	22%	SYHG	SYHG	SYHG	SYHG	0,675	1,988
	GL Python	1,2/240	0,52/13000	*0,43/*54,17	26%	AYHR	AYHR	AYHR	AYHR-AYHG	0,357	0,25
	Al Python	49/2500	2100/13000	*42,86/*5,2	20%	AYHR	AYHR	AYHR	AYHR	2,1	0,693
	Bl Python	1,2/2500	2,5/13000	*2,08/*5,2	2%	AYHR	AYHR	AYHR	AYHR	0,789	0,372
	Cl Python	2,5/500	260/13000	*104/*26	26%	AYHR	SYHG-AYHR	SYHG-AYHR	SYHG-AYHR	1,058	0,345
	AL Sophia	0,87/890	13/1100	*14,94/*1,23	24%	SYHG	SYHG	SYHG	SYHG	1,987	2,594
	BL Sophia	83/180	160/13000	*1,93/*72,22	6%	SYHG	SYHG	SYHG	SYHG	1,051	1,895
	GL Sophia	3,5/2500	0,11/100	*0,03/*0,04	4%	AYHR	/	AYHR	AYHR	0,016	0,608
	Al Sophia	6,1/2500	1200/13000	*196,72/*5,2	32%	AYHR	AYHR	AYHR	AYHR	0,699	0,884
	Bl Sophia	1,3/1400	300/13000	*230,77/*9,28	12%	AYHR	AYHR	AYHR	AYHR	0,041	0,198
	Cl Sophia	0,64/1400	3,1/13000	*4,84/*9,28	6%	AYHR	AYHR	/	AYHR-AYHG	0,332	0,193
	CL Julietta	<0,11/130	<0,11/1800	*1/*13,85	0%	/	/	/	/	0,006	0
	DL Julietta	280/13	160/5800	*0,57/*446,15	6%	SYHG-AYHR	AYHR	Undeterminated	AYHR	0,081	0,82
	EL Julietta	4,6/680	17/270	*3,69/*0,4	42%	AYHR	AYHR	AYHR	AYHR	0,326	0,326
	FL Julietta	<0,023/200	17/4700	*739,13/*23,5	0%	AYHR	AYHR	Undeterminated	/	0,322	0,18
	CL Bering	<0,11/390	<0,11/13000	*1/*33,33	0%	/	/	/	/	0,006	0
	DL Bering	0,13/1200	0,26/5800	*2/*4,83	2%	SYHG-AYHR	AYHR	/	AYHR	0,023	0,209
	EL Bering	3/230	3,9/2100	*1,3/*9,13	0%	AYHR	AYHR	AYHR	AYHR	0,481	0,52
	FL Bering	0,023/240	10/1200	*434,78/*5	0%	AYHR	AYHR	AYHR	AYHR	0,393	0,173
	CL Bison	<0,11/13000	<0,11/13000	*1/*1	0%	/	/	/	/	0	0
	DL Bison	2500/680	1200/13000	*0,48/*19,11	6%	SYHG-AYHR	AYPR-AYHR	AYHR	AYHR-AYPR	0,05	0,463
	FL Bison	0,023/88	1,5/13000	*65,22/*147,73	0%	AYHR	AYHR	Undeterminated	AYHR-AFHG	0,089	0,37
	EL Adriana	2,1/55	43/13000	*20,48/*236,36	0%	AYHR	AYHR	AYHR	AYHR	0,752	0,563
2011	AL Britta	ND	ND	ND	9%	ND	ND	SYHG-SYHN	SYHG	2,06	1,644
	BL Britta	ND	ND	ND	4%	ND	ND	SYHG-AYHR	SYHG-AYHR	0,46	0,461
	GL Britta	ND	ND	ND	7%	ND	ND	SYHG-SFHG-AFHR-AYHR	SYHG-AYHR	2,229	0,688
	Al Britta	ND	ND	ND	0%	ND	ND	AFLG-SYHG-AYHG-AYHR	SYHG-AYHR-mult	0,217	0,259
	Bl Britta	ND	ND	ND	10%	ND	ND	AYHR-TYHR	AYHR-TYHR	0,773	0,234
	Cl Britta	ND	ND	ND	16%	ND	ND	SYHG-AYHR	AYHG-SYHG-AYHR-SYHR	2,324	1,861
	AL Python	ND	ND	ND	27%	ND	ND	SYHG	SYHG-SYHR	2,545	1,334
	GL Python	ND	ND	ND	27%	ND	ND	SYHG-SCHG	SYHG-AYHR	2,286	1,153
	Al Python	ND	ND	ND	3%	ND	ND	AYHR	AYHR-SYHG	0,633	0,099
	Bl Python	ND	ND	ND	53%	ND	ND	AYHR	AYHR	1,131	0,442
	Cl Python	ND	ND	ND	10%	ND	ND	SYHG-AYHR	SYHG-AYHR	1,286	1,404
	AL Sophia	ND	ND	ND	19%	ND	ND	SYHG-SYHR	SYHG-AYHG-AYHR-SYHR	3,077	1,94
	GL Sophia	ND	ND	ND	23%	ND	ND	SYHG-AYHR	SYHG-AYHR	2,475	1,263
	Al Sophia	ND	ND	ND	8%	ND	ND	AYHR-SYHG	AYHR	1,111	0,205
	Bl Sophia	ND	ND	ND	40%	ND	ND	AYHR	AYHR	1,511	0,518
	Cl Sophia	ND	ND	ND	7%	ND	ND	SYHG	AYHG-SYHG-AYHR-SYHR	2,512	1,709
	CL Julietta	ND	ND	ND	2%	ND	ND	SYHG-AYHR-AFHR	SYHG-AYHR	0,612	0,316
	DL Julietta	ND	ND	ND	4%	ND	ND	SYHG-AYHR	AYHR-SYHG	1,278	0,752
	EL Julietta	ND	ND	ND	3%	ND	ND	AYHR-SYHG-SFHG	AYHR	0,779	0,934
	FL Julietta	ND	ND	ND	18%	ND	ND	AYHR	AYHR	1,772	0,416
	CL Baobab	ND	ND	ND	0%	ND	ND	SYHG	AYHR-SYHG	0,205	0,383
	DL Baobab	ND	ND	ND	6%	ND	ND	AYHR	SYHG-AYHR	0,613	0,294
	EL Baobab	ND	ND	ND	14%	ND	ND	AYHR	AYHR	0,462	0,639
	FL Baobab	ND	ND	ND	13%	ND	ND	AYHR	AYHR	1,626	0,757
	CL Bison	ND	ND	ND	1%	ND	ND	SYHG-VYHG	SYHG-AYHR	0,417	0,496
	DL Bison	ND	ND	ND	6%	ND	ND	AYHR	AYHR	0,543	0,346
	EL Bison	ND	ND	ND	17%	ND	ND	AYHR	AYHR	1,041	0,719
	FL Bison	ND	ND	ND	46%	ND	ND	AYHR	AYHR	1,582	0,36
	BL Rosalinda	ND	ND	ND	11%	ND	ND	SYHG	SYHG	1,059	0,658
	BL Magellan	ND	ND	ND	19%	ND	ND	SYHG	SYHG	1,842	1,144

Rem: The virus and vector MPN for May 2010 and for September 2010 are shown for each varietal band. The multiplication rate between May 2010 and September 2010 per varietal band is shown. The root severity in September is reminded as the tetrad in May and September in the bioassays 2010 and the assays 2010 and 2011.

4.9. How the different varieties used behave in different soils contexts? Is there a varietal effect on the infectious potential during the beet season?

The table above (Table 4.1) presents the virus MPN and the vector MPN before the beginning of the beet culture and in the period of the harvest. The multiplication rate during the beet season is also indicated. The root severity is reminded together with the tetrad in May and September in the bioassay and from the field samples. All the results are classified by varieties used in the assay in the field. The average per varieties is presented in the table 4.2. Overall, there was an increase of the MPN between the beginning of the beet culture and the harvest period (Table 4.2). The relation was not clear between the MPN values and the root severity when the variety "Julietta" was looked. But, the root severity for "Python" and "Sophia" was linked to the virus MPN. The variety "Python" did not have a good behaviour against the virus while this variety was considered as a *Rz1* partial resistant variety with good resistance. However, there was MPN variability between each different variety that could be due to a different genetic background and varietal pressure in the field. But, it was known since the beginning that these results depended also of a high intra-plot and inter-plot variability due to the distribution in the soil. We must keep also in mind that the analysis is made on dried soils. It could have a potential effect on the nematodes and consequently on the virus also in the bioassay level in case of synergism. Consequently, there was not a clear relation between the disease measure indicators and the root severity which is the disease impact indicator in the field.

Table 4.2. Average of the comparison table between infectious potential (virus MPN & vector MPN) before the beginning of the beet culture and in the moment of beet harvest in 2010 per variety.

PI average per variety				
Variety	IP May /variety band (MPN virus/MPN vector)	IP Sept. /variety band (MPN virus/MPN vector)	Muttiplication rate in the band	Root gravity in September
Ludwinia	22/697	70/9068	*3/*13	6%
Python	10/1110	402/11133	*40/*10	21%
Sophia	16/1478	279/8867	*17/*6	14%
Julietta	71/256	48/3142	*0,68/*12	12%
Bering	0,81/515	3,6/5525	*4/*12	0,50%
Bison	833/4589	400/13000	*0,48/*3	2%

Rem: The multiplication rate between May and September per variety is shown. The virus and vector MPN for May and for September are shown for each variety. The root severity in September is reminded.

When the virus MPN in May and September was compared for the evolution in the sugar beet season, the level of virus MPN in September was

explained for 58,08% by the virus MPN in May (Figure 4.7). There was a link between the MPN in May and September. A clear effect of the variety on the virus MPN, however, was here not observed.

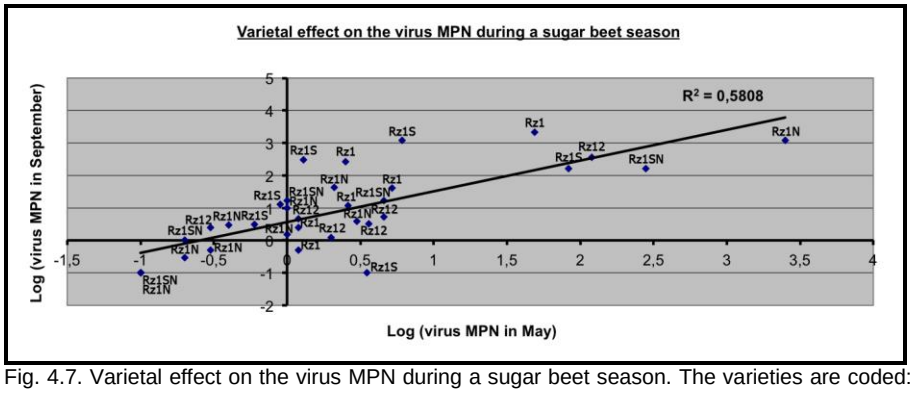


Fig. 4.7. Varietal effect on the virus MPN during a sugar beet season. The varieties are coded: *Rz1*S for *Rz1* cultivars with weak resistance, *Rz1*SN for *Rz1* cultivars with weak resistance and nematode tolerance, *Rz1* for *Rz1* cultivars with good resistance, *Rz1*N for *Rz1* cultivars with good resistance and nematode tolerance, *Rz12* for cultivar with *Rz1* and *Rz2* genes.

The figure 4.8 shows the results for the comparaison of the vector MPN in May and September. Compared to the virus MPN, the level of MPN was higher. Unfortunately, due to the analysis protocol, threshold for the detection of the vector was achieved. In conclusion, there was for some samples at least 13000 infectious unit of vector per gram of soil, but maybe more in fact. And for these samples, the level could be much higher.

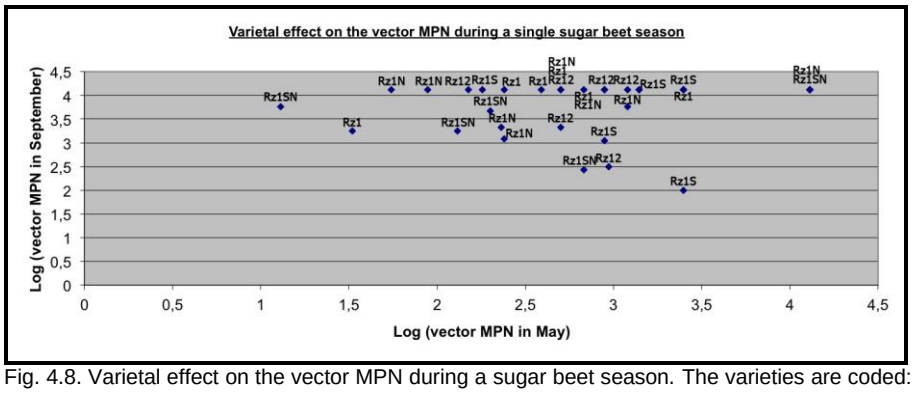


Fig. 4.8. Varietal effect on the vector MPN during a sugar beet season. The varieties are coded: *Rz1*S for *Rz1* cultivars with weak resistance, *Rz1*SN for *Rz1* cultivars with weak resistance and nematode tolerance, *Rz1* for *Rz1* cultivars with good resistance, *Rz1*N for *Rz1* cultivars with good resistance and nematode tolerance, *Rz12* for cultivar with *Rz1* and *Rz2* genes.

To calculate the viruliferous vector, we must keep in mind here that for some samples, the level should be lower due to the threshold of vector MPN. Nevertheless, the figure 4.9 shows that the viruliferous vector in September was explained for 32,71% by the viruliferous vector in May. There was a link between the results in May and September. But, the effect of the varieties used is here not clear.

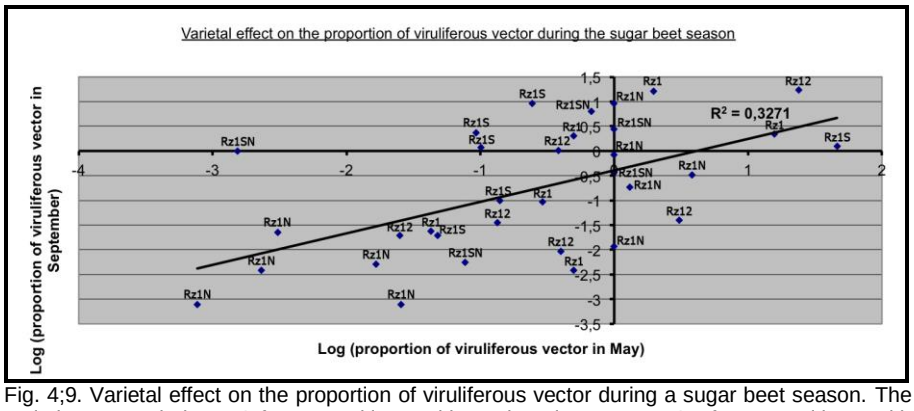


Fig. 4;9. Varietal effect on the proportion of viruliferous vector during a sugar beet season. The varieties are coded: *Rz1*S for *Rz1* cultivars with weak resistance, *Rz1*SN for *Rz1* cultivars with weak resistance and nematode tolerance, *Rz1* for *Rz1* cultivars with good resistance, *Rz1*N for *Rz1* cultivars with good resistance and nematode tolerance, *Rz1*2 for cultivar with *Rz1* and *Rz2* genes.

For some samples with low level in May, we observed high level in September (upper left section in Figure 4.9). For some samples with high level in May, we observed high level in September (upper right section). For other samples with high level in May, we observed (lower right section). The majority of the samples showed low level of viruliferous vector in May and in September (lower left section).

4.10. Is the tetrad found in the field sample linked to the infectious potential of the soil?

The highest ELISA values for the virus were linked to the SYHG tetrad in the roots from the field. The SYHG tetrad, however, was less present in relation with the vector than the AYHR tetrad. The figure 4.10 shows that the highest virus and vector MPN were found for the AYHR tetrad detected in the assays from the field. The highest different points in the vector MPN corresponded to the detection threshold of the vector by the MPN technique from the bioassays.

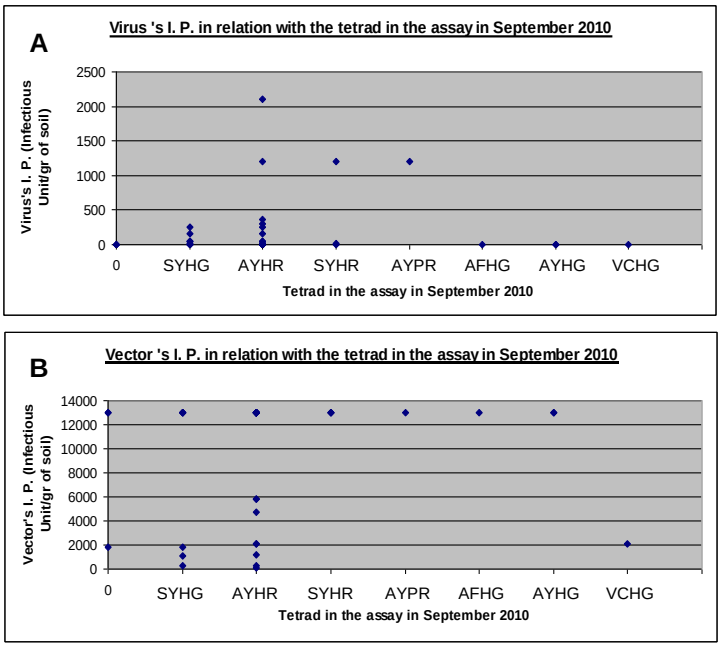


Fig. 4.10. Virus (A) and vector (B) MPN (infectious potential) in relation with the tetrad in the assay in September 2010.

4.11. Is the tetrad found in the field sample linked to the proportion of viruliferous vector in the soil?

The results showed that the proportion of viruliferous vector in the soil in the beginning of the culture was in a wider range of value for the tetrad SYHG detected in the root samples compared to the tetrad AYHR of samples in mixed infection (Figure 4.11).

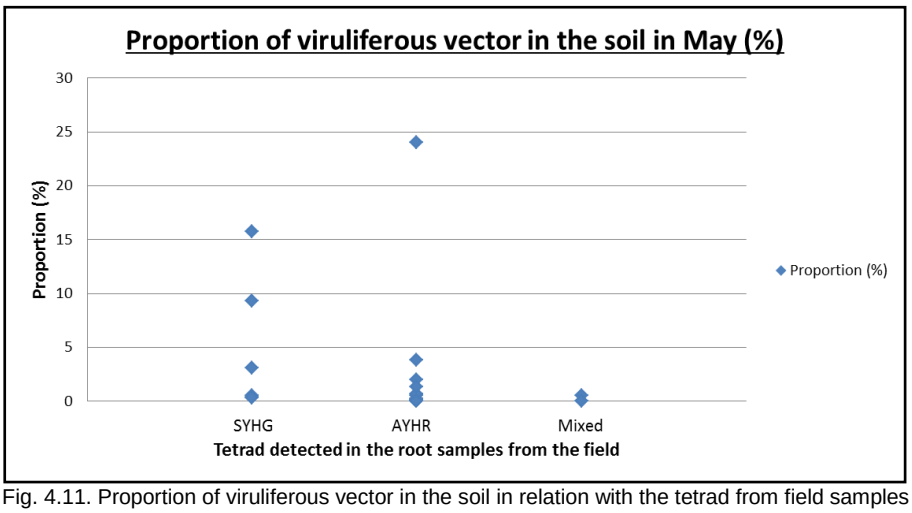


Fig. 4.11. Proportion of viruliferous vector in the soil in relation with the tetrad from field samples in May.

The proportion of viruliferous vector in the soil, however, was in a wider range of value for the tetrad AYHR at the end of the sugar beet season compared to the tetrad SYHG (Figure 4.12).

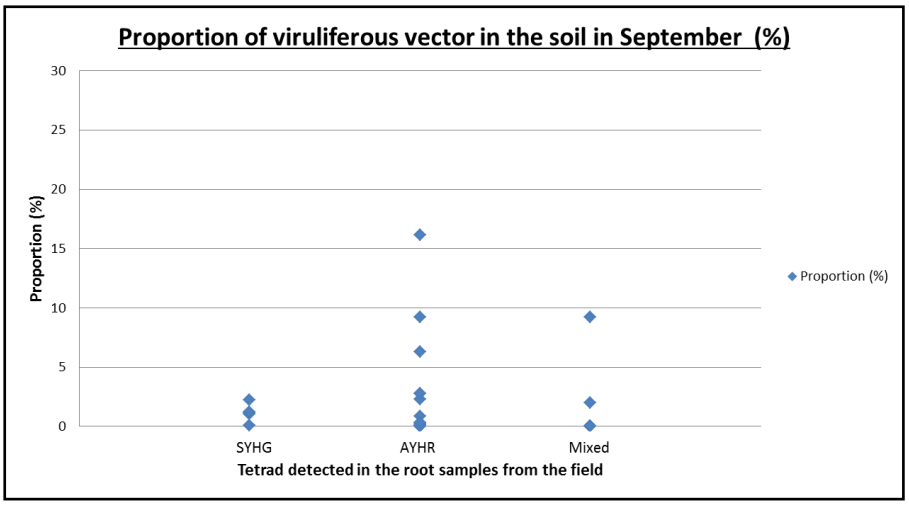


Fig. 4.12. Proportion of viruliferous vector in the soil in relation with the tetrad from field samples in September.

This indicates that the proportion of viruliferous vector was higher in the soil for the tetrad SYHG before the implementation of sugar varieties in the field. And during the sugar beet season, the proportion of viruliferous vector linked to the tetrad SYHG decreased in the soil due to the sugar beet culture. The proportion of viruliferous vector linked to the tetrad AYHR, however, increased in the soil with the sugar beet culture.

4.12. Is there a varietal effect on the virus?

The figure 4.13 shows differences in the quantity of the tetrad SYHG and AYHR and also in the ELISA values according to the different varieties tested in September 2010. The figure shows firstly that the double resistance contained more double infection of pathotype in a single beet root. There might had a higher selection pressure or the variety allowed the presence of several tetrads within the sugar beet. Secondly, the virus content in the variety Ludwina was lower than the content in the variety Python and much lower than the content in the variety Sophia which was the *Rz1* cultivar with weak resistance. If we have several pathotypes in a variety, however, there could had a reassortment between the BNYVV B-pathotype and P-pathotype. What we didn't know was if the virus was developing in different cells or in similar cells. What happen when the virus come in the same cell of the beet root? What we only observed in general was that the double resistance varieties were infected by more diversified pathotype of BNYVV than the others beet varieties. But, when there was simple infection of SYHG, the ELISA values were higher than in mixed infection with AYHR in

the several varieties. Has the tetrad AYHR in pre-infection or mixed infection the capacity to inhibit the SYHG content and the high ELISA values linked to the tetrad SYHG? The double resistance sugar beet allowed also mixed infection. When there was simple infection the AYHR content or the SYHG content was higher. The partial resistant variety carrying the *Rz1* resistant gene allowed more simple infection and consequently higher BNYVV content in the plant. Another important observation of this figure was the fact that the varieties used in fields with nematode were varieties with resistance gene against the nematode and the BNYVV (Adriana, Bering, Bison and Julietta). All the fields infested by nematodes and investigated were mainly found with the AYHR tetrad but less with the SYHG tetrad.

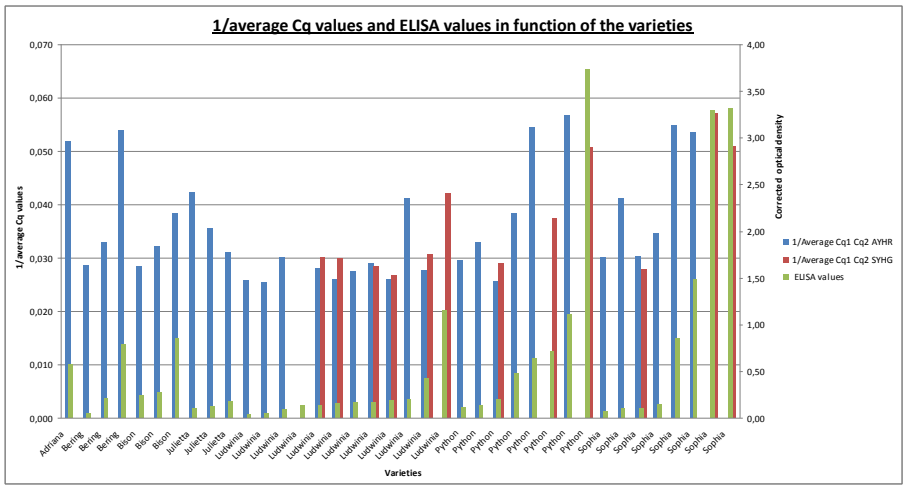


Fig. 4.13. Inverse of the average values Cqnorm and ELISA values (corrected OD) as a function of the varieties used. Presence and quantity of the two tetrads AYHR (blue) and SYHG (red), and the respective ELISA values (green) of 38 single root samples of sugar beet from fields in September 2010 are presented. The results are classified firstly by varieties and secondly by ascending values of ELISA.

If we normalized with the ALS gene (linearization) and showed the results in term of quantitative results Rq (ng/µl) rather than Cq, we should had a better representation of the relative quantity of tetrad SYHG and AYHR in each variety (Figure 4.14). The ALS reference gene, however, was so expressed that the Cq values of ALS reference gene overrided the Cq values of the tetrads. The relative representation was not directly clearly visible. The results had been consequently transformed for a better representation.

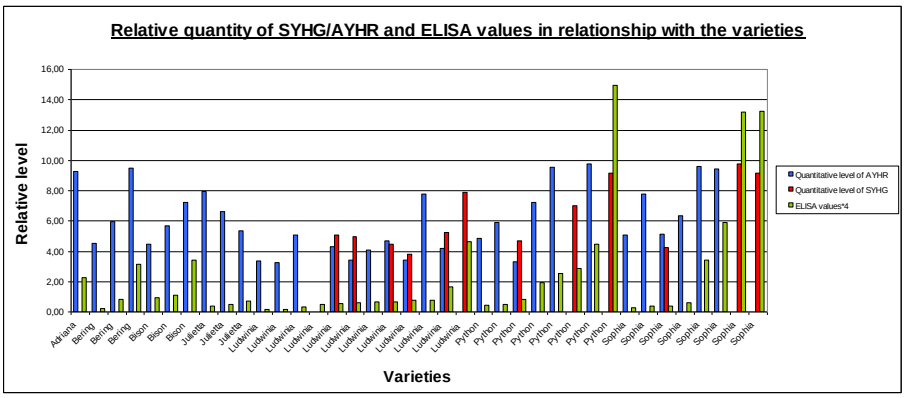


Fig. 4.14. Correlation between the quantitative results (Rqnorm) (ng/µl) of SYHG and AYHR, the ELISA values (corrected OD) and the sugar beet varieties used of 38 samples from September 2010. For better representation, the level of ELISA values is here multiplied by four. The Rq values normalized are transformed by the logarithm to show the multiplication factor.

The figure 4.15, 4.16 and 4.17 shows the ELISA values of BNYVV in different varieties obtained for 480 samples from May 2010 and 2011 and September 2010 and 2011. The variety Python (*Rz1* cultivar with good resistance) showed the highest ELISA values. This variety did not have a good behavior against the virus. Even, this variety had a worse behavior than the variety Sophia (*Rz1* cultivar with weak resistance) which showed also high ELISA values. The double resistance varieties had also a wide range of ELISA values but in a slightly level behind Python and Sophia. The varieties resistant against the BNYVV and the nematodes had a better behavior against the virus level in the sugar beet. The figure 4.18 represents the results for each field in 2010 and in 2011. In this way, we can compare the behavior of each variety in the same soil context.

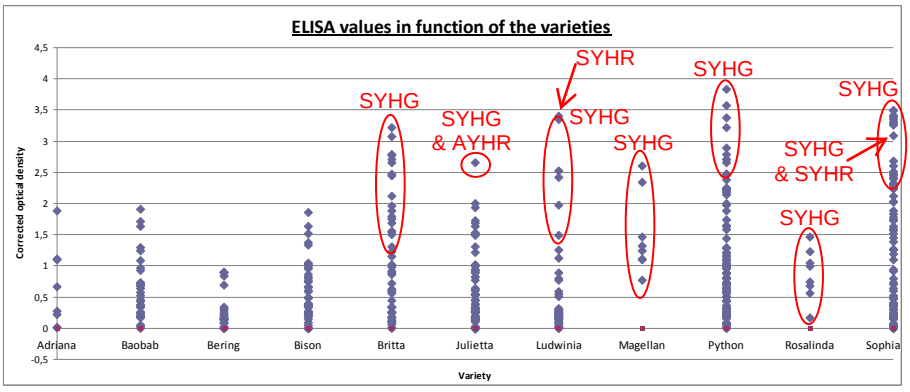


Fig. 4.15. Correlation between the ELISA values (corrected OD) and the sugar beet varieties used of 480 samples from May 2010 and 2011 and September 2010 and 2011.

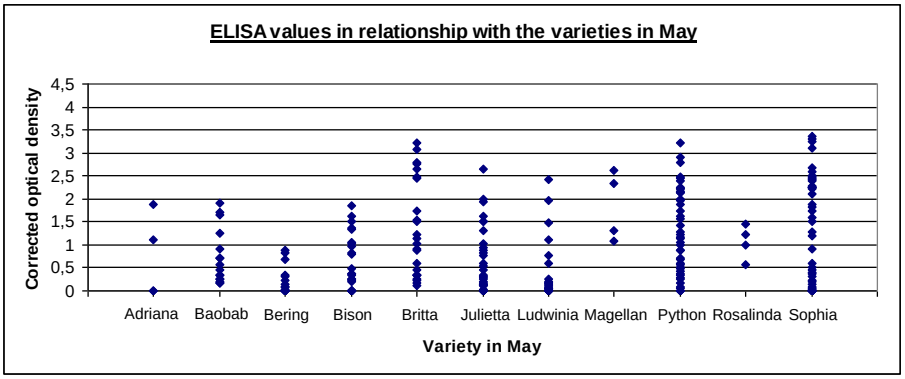


Fig. 4.16. Correlation between the ELISA values (corrected OD) and the sugar beet varieties used of 240 samples from May 2010 and 2011.

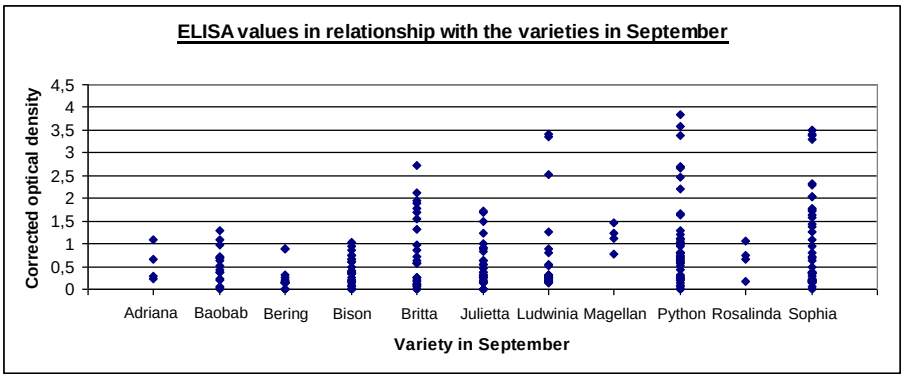
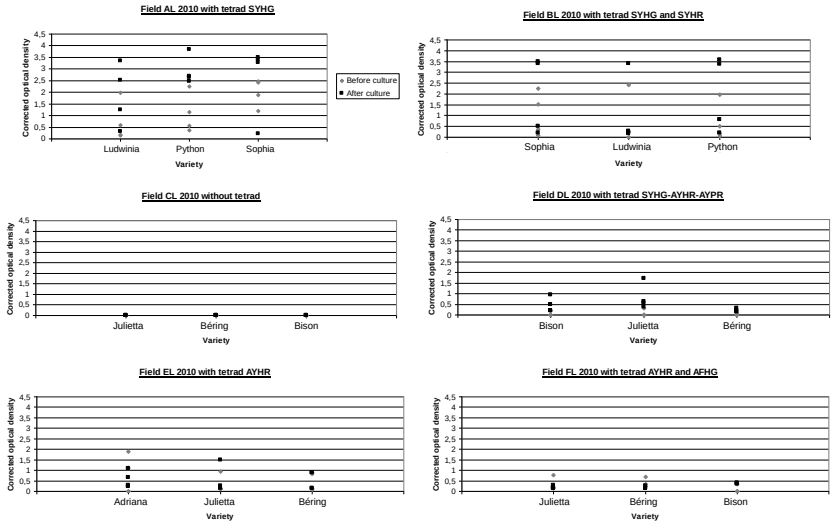


Fig. 4.17. Correlation between the ELISA values (corrected OD) and the sugar beet varieties of 240 samples from September 2010 and 2011.



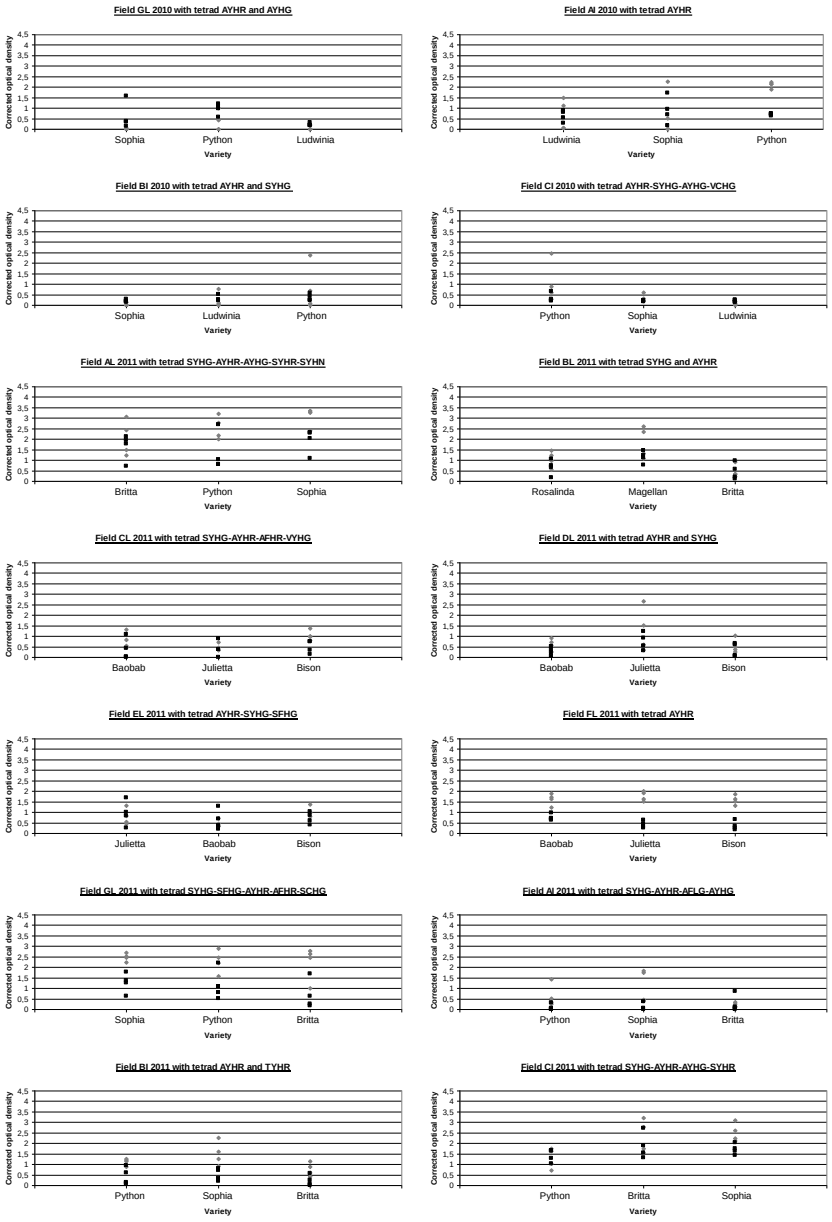


Fig. 4.18. Correlation between the ELISA values (corrected OD) and the sugar beet varieties used in May and September in each field analyzed in 2010 and 2011. In this way, there is the same soil context.

The figure 4.19 to 4.22 represents respectively the ELISA values in May 2010 September 2010, May 2011 and September 2011. The results were classified by variety and the origin of the variety in the field was reminded. The figures 4.19 and 4.20 show also that the variety Python was not a good *Rz1* partial resistant variety (wide range of high ELISA values) and was

worse than the variety Sophia considered as *Rz1* cultivar with weak resistance. The *Rz1Rz2* variety had a better effect on the BNYVV level than the variety Python and Sophia. The lowest ELISA values were still obtained by the cultivar with virus and nematodes resistance. It was less the case in 2011 (figure 4.21 and 4.22). In 2011, the BNYVV level was higher than in 2010. The *Rz1Rz2* varieties had a wider range of ELISA values and the varieties resistant against BNYVV and the nematodes also.

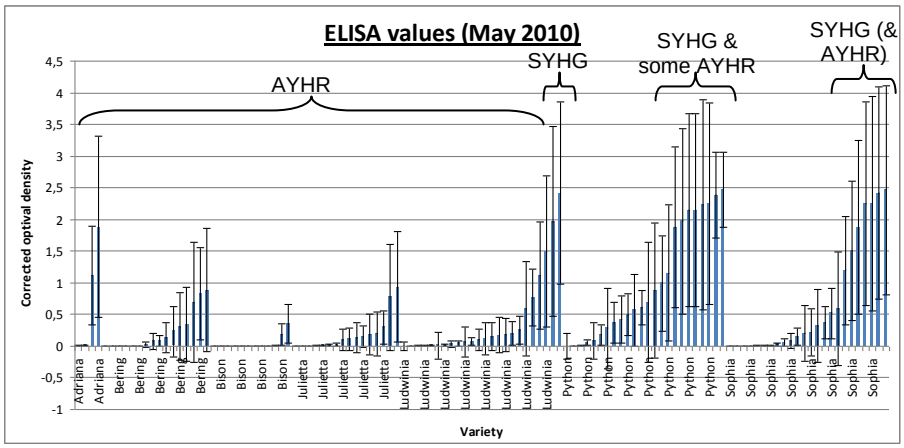


Fig. 4.19. Corrected optical density values obtained by DAS-ELISA targeting the CP protein coded by the BNYVV RNA2 of 120 samples in May 2010. The name of sugar beet varieties used is indicated in abscises. The samples are classified by sugar beet varieties and by ascending ELISA values.

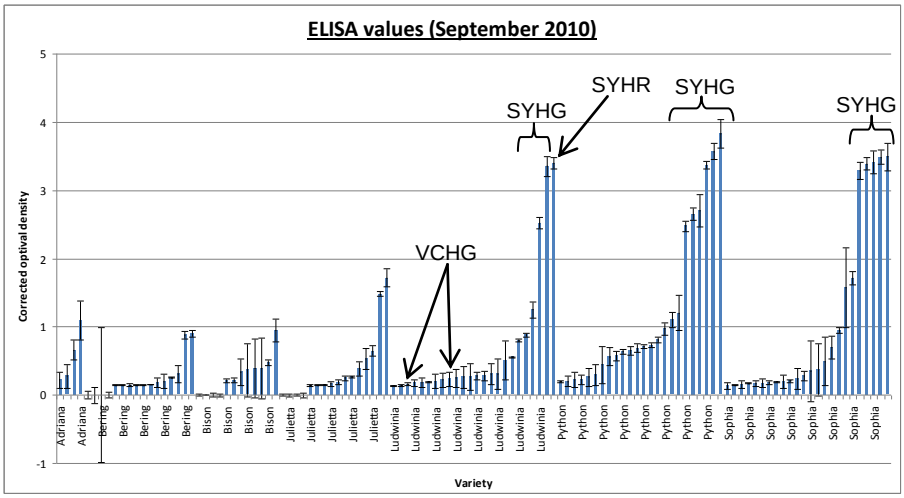


Fig. 4.20. Corrected optical density values obtained by DAS-ELISA targeting the CP protein coded by the BNYVV RNA2 of 120 samples in September 2010. The name of sugar beet varieties used is indicated in abscises. The samples are classified by sugar beet varieties and by ascending ELISA values.

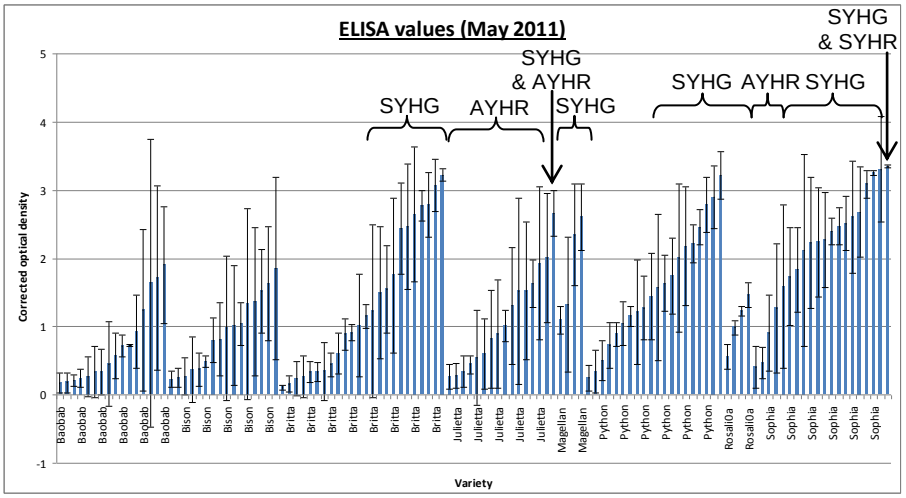


Fig. 4.21. Corrected optical density values obtained by DAS-ELISA targeting the CP protein coded by the BNYVV RNA2 of 120 samples in May 2011. The name of sugar beet varieties used is indicated in abscises. The samples are classified by sugar beet varieties and by ascending ELISA values.

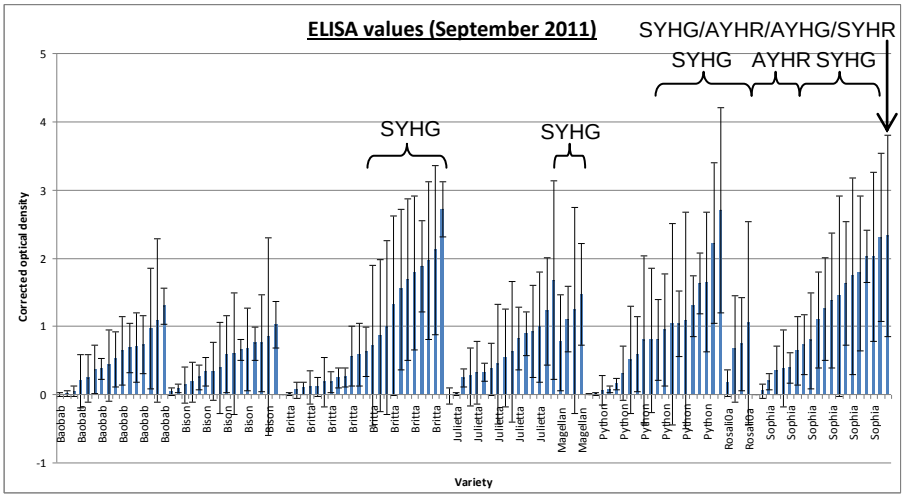


Fig. 4.22. Corrected optical density values obtained by DAS-ELISA targeting the CP protein coded by the BNYVV RNA2 of 120 samples in September 2011. The name of sugar beet varieties used is indicated in abscises. The samples are classified by sugar beet varieties and by ascending ELISA values.

4.13. What's the ELISA evolution during the sugar beet season?

The figure 4.23 and 4.24 show the comparison between the results obtained in May and September. These figures show the evolution during the sugar beet season in all the fields investigated. Overall, for all the varieties in 2011, a decrease of the ELISA values during the sugar beet season was observed. The resistance gene increased its efficacy during the season with the growth

of the sugar beet. It was, however, the opposite in 2010. In 2010, the varieties were not really affected by the BNYVV. The spring was to dry for the implementation of the disease. Indeed, in May 2010, there were few symptoms and the weather was hot and dry. In September, however, there was an increase of the precipitation. The figure 4.23 shows that the BNYVV level was higher in September. In 2011, the varieties were more affected by the virus and there were more symptoms in the field. In May 2011, the weather was wet. The vector and virus implemented well. The figure 4.24 showed higher BNYVV level in May 2011. The symptoms observed in the fields were linked to the good installation of the virus in the sugar beet early in the season and also linked to the weather conditions. There was a decrease in the CP production (ELISA) during the sugar beet season. The efficiency of the resistance increased with the age of the plants even the symptoms were present in September.

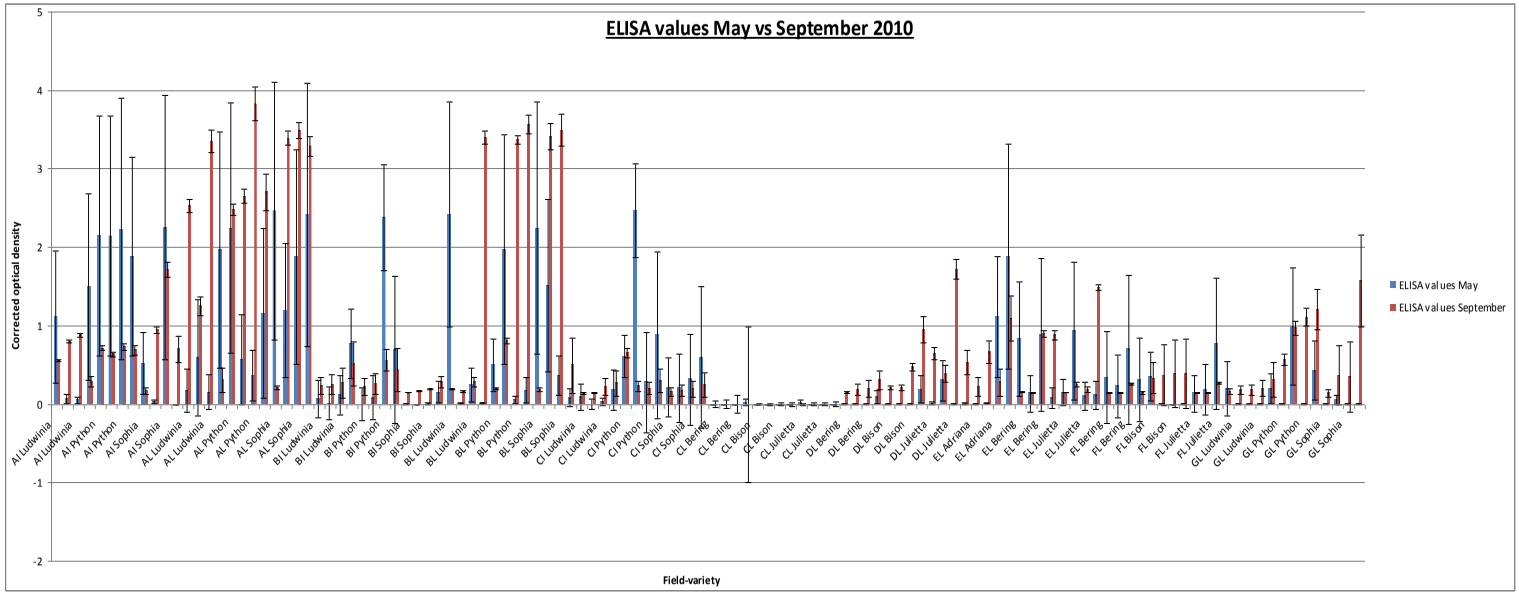


Fig. 4.23. Comparison of the ELISA values (corrected OD) in May and September 2010 in function of the fields and the varieties used of 240 samples from May and September 2010. The two first letters correspond to the field where the samples were obtained and are followed by the variety name of the sugar beet used. Each couple of histogram represents a sample couple, sampled either in May either in September in the same location in the field.

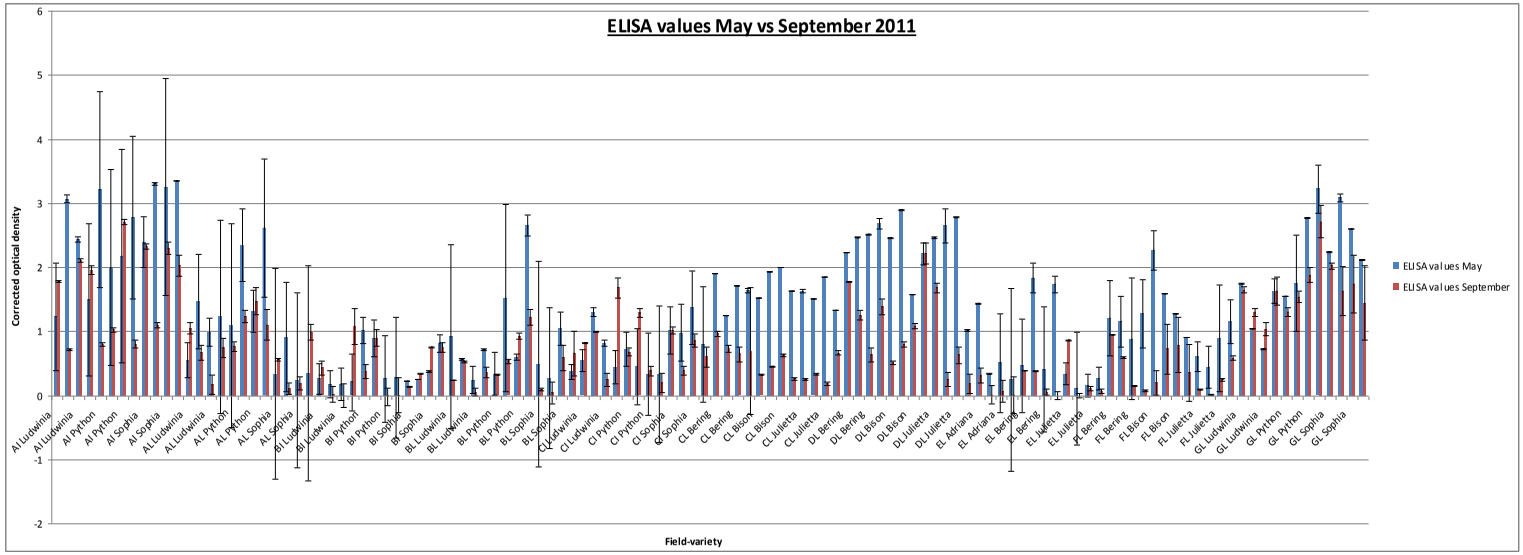


Fig. 4.24. Comparison of the ELISA values in May and September 2011 in function of the fields and the varieties used of 240 samples from May and September 2011. The two first letters correspond to the field where the samples were obtained and are followed by the variety name of the sugar beet used. Each couple of histogram represents a sample couple, sampled either in May either in September in the same location in the field.

4.14. The RT-mqPCR results performance compared to the ELISA values

The figure 4.25 shows the good correlation between the ELISA data and the real time RT-mqPCR results. This graph was clearly important because we could expect divergences between the two analyses. Indeed, the ELISA dosed the CP protein producted by the BNYVV RNA2 while the RT-mqPCR dosed the BNYVV RNA3 presence.

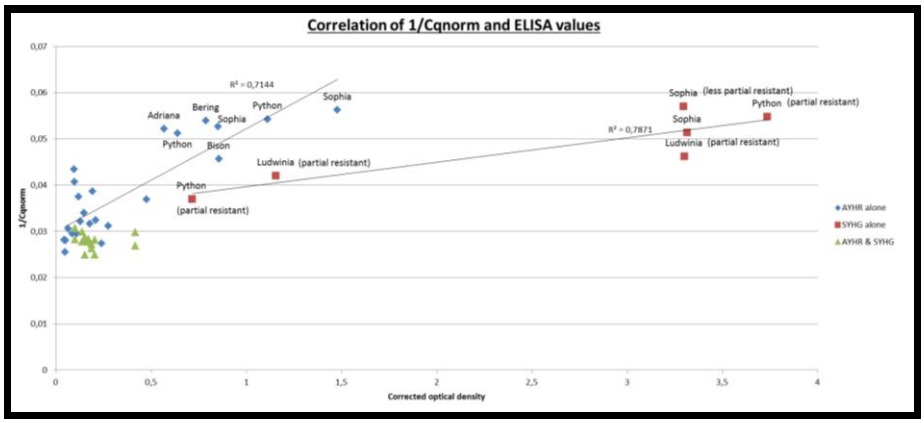


Fig. 4.25. Correlation of the inverse of average values of Cq and ELISA values (corrected OD). It is the correlation between the 1/Cqnorm of SYHG alone (red square), AYHR alone (purple diamond), SYHG in presence of AYHR (green triangle) and the corrected optical density values (ELISA) of 46 single root samples of sugar beet from the field in September 2010. For SYHG alone, the higest Python is linked to 32% of root severity in the varietal band. The lowest Python is linked to 22% of root severity in the varietal band. For Ludwina, there is 10% of root severity and for Sophia there is 6% of root severity in the varietal band.

What was important behind these results was the genetic background of the plants. In analyzing the data, we noted that the three points with the highest ELISA values corresponding to two varieties “Sophia” (*Rz1* cultivar with weak resistance) and one variety “Python” (*Rz1* cultivar with good resistance, but finally with a weak resistance). Moreover, the SYHG alone points corresponded to the ELISA values basis and corresponded to the variety Ludwina. The double resistance variety decreased the content of the P-pathotype. This graph shows also that the AYHR tetrad was often more present in the different samples analyzed compared to the SYHG tetrad. There was, however, a better correlation between the average 1/Cq for SYHG and the ELISA values than the average 1/Cq for AYHR. Indeed, the R^2 (determination coefficient) showed that the increase of the average 1/Cq values for SYHG alone was explained for 78,71% by a higher ELISA value while for AYHR alone it was explained only for 71,44%.
The figure below (Figure 4.26) shows that the highest ELISA values corresponded essentially to the BNYVV P-pathotype. For these three ELISA values (SYHG alone), they were found in two *Rz1* cultivars with weak resistance and one *Rz1* cultivar with good resistance. The two foregoing values of SYHG alone which were three times lower were found in the *Rz1Rz2* cultivars. The ELISA values found between 0,099 and 0,241

corresponded to mixed infection of pathotype (AYHR and SYHG) and were mainly present in the *Rz1Rz2* cultivars. These double resistant varieties allowed mixed infection of BNYVV pathotype and might also increase the occurrence of reassortiment/recombination between the two pathotypes. There was a selection pressure of this double resistant variety. This could lead to the appearance of new mutants responsible of resistance-breaking in the plant. Moreover, the level of SYHG was inhibited in mixed infection of pathotype in the double resistant plant. To conclude this, there was high ELISA values in *Rz1* partial resistant genotype with weak resistance in presence of SYHG alone, low ELISA values in *Rz1Rz2* partial resistant genotype in presence of SYHG alone and mixed infection of pathotype.

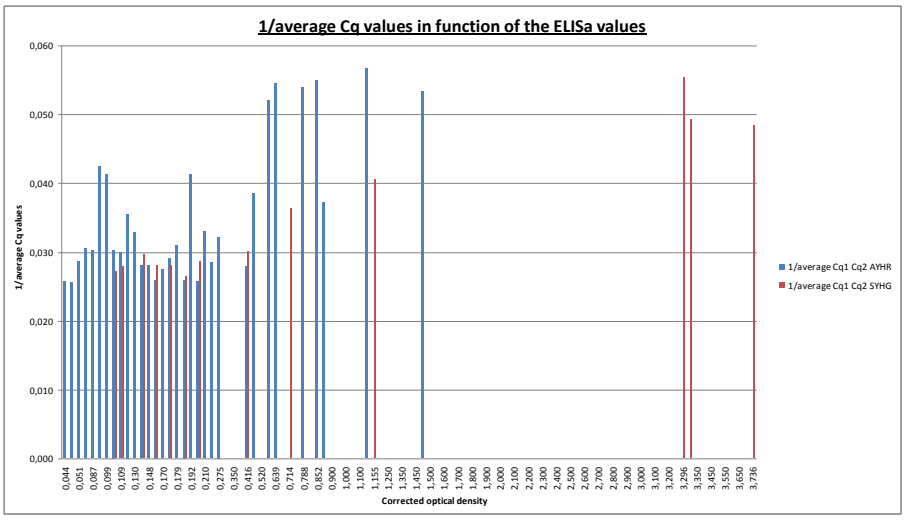


Fig. 4.26. Inverse of average values of Cq as a function of ELISA values (corrected OD). The figure represents histogram of 1/Cq values of the SYHG and AYHR tetrad as a function of the corrected optical density of BNYVV.

The figure 4.27 was interesting because it showed that the real time RT-mqPCR detected directly the presence and the proportion of each BNYVV pathotype especially in case of mixed infection while the direct sequencing detected only the major pathotype. The figure 4.27 shows that the multiplex RT-mqPCR works quite well to detect the two BNYVV pathotype in comparison with the sequencing results. The real time RT-mqPCR was even better to detect double infection in a single beet root while the sequencing detected the major pathotype. This was showed for the tetrad SYHG and AYHR in simple infection when it was detected by direct sequencing. The question which also emerged from these data was the possibility of a reassortiment between BNYVV B-/P-pathotypes.

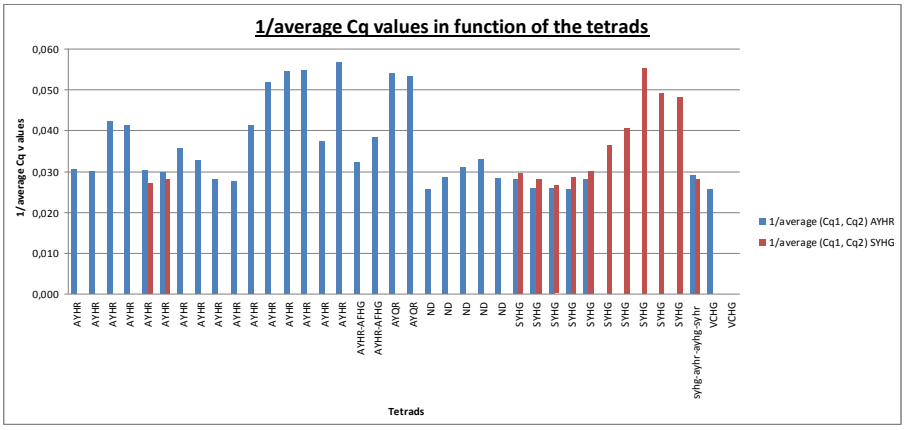


Fig. 4.27. Inverse of average values of Cq as a function of the tetrads detected in the assays implemented in ten different fields in September 2010.

The figure 4.27 shows also that some samples were identified with a single tetrad by direct sequencing while mixed tetrad by RT-mqPCR. Indeed, the direct sequencing shows only the major pathotype present in the sample. The detection by duplex RT-mqPCR was more accurate. The figure shows, in general, that the sample in mixed infection with AYHR and SYHG were in lower content compared to simple infection with AYHR or SYHG. The two tetrads were in competition in a single sugar beet root.

4.15. What is the difference between the A-, B- and P-pathotype in the BNYVV RNA3?

The table 4.3 shows the comparison between the pathotypes with the tetrad TYHR, AYHR, SYHG and AYPR. The table shows the aa mutation between each pathotype and also for the nucleic acid mutation. The silent mutations can also be seen. There were some positions in the mutation that were more important because there were more differences between each pathotype in the silent mutation and in the visible mutation. When the table 4.3 and the silent mutation were looked and when the three pathotypes between them were compared, there were acid nucleic mutations. The position of the aa mutated in the p25 due to these acid nucleic mutations were in the position 21, 23, 28, 30, 32, (37), 42, 49, (55), (63), 67, 68, 69, 70, 73, (88), (101), (102), (115), [118], 129, 132, (142), (149), [163], 179, (186), 192, 198, 213 and 219 of the p25 (aa in () corresponded to silent mutation in the genetic code and [] was when there was two acid nucleic mutations for one aa in the genetic code). In France, the five aa mutations which distinguished the P-pathotype from the two others were in the position 28 (T), 67 (S), 129 (N), 163 (S) and 198 (A). The amino acids which were the same for the B-pathotype and the P-pathotype, but different for the A-pathotype, were in the position 49 (S) and 132 (I). The five aa mutations which distinguished the B-pathotype from the two others were in the position 32 (N), 118 (T), 129 (D), 179 (D) and 192 (I).

There was consequently a need to quantify these different pathotypes in a single beet to know the importance of each one and to have the possibility to see if there was a varietal effect from the beet and a selection pressure on the virus BNYVV (data not shown here).

Table 4.3. Amino acid and acid nucleic mutation position comparison in the p25 of five tetrad strains of the RNA3 from the BNYVV in the region of Pithiviers.

A. a. mutation position:	27	28	30	32	34	37	42	47	49	55	62	63	67	68	69	70	86	98	101	102	106
Amino acid Tetrad																					
AYHR	maj L/1S	I	*S*	N	maj N/1D	*R*	L	*R*	S	*N*	maj R/1C	*G*	A	Y	H	R	*L*	maj R/1C	*V*	*A*	maj R/1T
TYHR	L	I	*S*	N	N	*R*	L	*R*	S	*N*	R	*G*	T	Y	H	R	*L*	R	*V*	*A*	R
SYHG	maj L/1S	maj T/2P	*S*	H	N	*R*	L	*R*	S	*N*	R	*G*	S	Y	H	G	*L*	R	*V*	*A*	R
AYPR	L	I	*S*	H	N	*R*	S	*R*	T	*N*	R	*G*	A	Y	P	R	*L*	R	*V*	*A*	R
Rem: AHHG	L	I	*S*	H	N	*R*	L	*R*	T	*N*	R	*G*	A	H	H	G	*L*	R	*V*	*A*	R

N. a. mutation position:	[80]	[82]	83	90	94	[100]	111	125	[141]	[145]	165	[184]	189	199	202	206	208	256	[292]	303	306	[317]
Nucleic acid Tetrad																						
AYHR:	maj T/1C	A	T	C	A	maj A/1G	*maj A/1G*	T	*maj T/1C*	T	*C*	maj C/1T	*T*	G	T	A	A	*T*	maj C/1T	*T*	*maj G/1A*	maj G/1C
TYHR:	T	A	T	C	A	A	*A*	T	*T*	T	*C*	C	*T*	**A**	T	A	A	*T*	C	*T*	*G*	G
SYHG:	maj T/1C	maj A/2C	**C**	**T**	C	A	*A*	T	*T*	T	*T*	C	*A*	**T**	T	A	**G**	*C*	C	**C**	*A*	G
AYPR:	T	A	T	C	C	A	***G***	**C**	*T*	**A**	*T*	C	*A*	G	T	**C**	A	*C*	C	*T*	*A*	G
Rem: AHHG	T	A	T	C	C	A	***G***	T	*T*	A	*T*	C	*A*	G	**C**	A	**G**	*C*	C	*T*	*A*	G

A. a. mutation position:	*114*	*115*	118		129	132	133	137		*142*	*149*	160	163		165	173	179	185	*186*	192	198	200
Amino acid Tetrad																						
AYHR	*L*	*L*	T		D	I	maj H/1P	maj F/1S		*F*	*P*	maj F/1S	A		maj T/1P	C/F/Y	D	E	*S*	I	T	D/N
TYHR	*L*	*L*	T		D	I	H	F		*F*	*P*	F	A		T	C	D	E	*S*	I	T	D
SYHG	*L*	*L*	N		N	I	H	F		*F*	*P*	F	S		T	C	N	maj E/1K	*S*	V	A	D
AYPR	*L*	*L*	N		H	L	H	F		*F*	*P*	F	P		T	C	D	E	*S*	V	T	D
Rem: AHHG	*L*	*L*	N		H	L	H	F		*F*	*P*	F	P		T	C	N	E	*S*	V	T	D

N. a. mutation position:	*[340]*	*343*	353	354	385	394	[399]	[410]	[411]	*426*	*447*	[479]	487	489	[493]	[518]	535	[553]	*558*	574	592	[598]
Nucleic acid Tetrad																						
AYHR:	*maj T/4C*	*T*	C	maj C/1T	G	A	maj A/1C	maj T/1C	idem	*T*	*T*	maj T/1C	C	G	maj A/1C	maj G/ 1T, 1A	G	G	*T*	A	A	maj G /2A
TYHR:	*T*	*T*	C	C	G	A	A	T	T	*T*	*T*	T	C	G	A	G	G	G	*T*	A	A	G
SYHG:	*T*	*C*	A	T	A	A	A	T	T	*T*	*C*	T	T	A	A	G	A	maj G/1A	*C*	G	G	G
AYPR:	*T*	*C*	A	T	C	T	A	T	T	*C*	*T*	T	C	A	A	G	G	G	*C*	G	A	G
Rem: AHHG	*T*	*C*	A	T	C	T	A	T	T	*C*	*T*	T	C	A	A	G	A	G	*C*	G	A	G

	Important mutation really distinct between different tetrad
	Some nucl. ac. Are different in a same tetrad
	Silent mutation and really distinct between different tetrad
	Silent mutation on some different nucleic acid in a same tetrad
[]	Minor mutations and then the others are major mutations between tetrad

Rem: On a total of 53 different strains with 13 SYHG, 1 TYHR, 1 AYPR, 1 AHHG et 37 AYHR

The main hypothesis was that there was a selection of a tetrad more aggressive and that the situation in the USA was different from that one in Europe. Indeed, in Europe, as it can see in the table 4.4, 6-7 systemic mutations were need to change from an A-pathotype to P-pathotype which was molecularly impossible. Ten systematic mutations were needed to change from an B-pathotype to P-pathotype which was also impossible. So what can be the likely path of change from a pathotype to another? If there was a mixture of the tetrad SYHG and AYPR, what can be the risk for the future? Was there frequent mixture of different isolates? In USA, Acosta-Leal described that they had only a single mutation transforming a wide type to a viruliferous type. Their situation was thus different compared to our situation.

For the eight fields analyzed, 70.37% of the virus showed the tetrad AYHR which was related to the B-pathotype. A new tetrad in the RNA3 from the BNYVV had also been identified and was TYHR. By phylogenicity, this tetrad was related to the B-pathotype. 24.79% of the virus detected showed the tetrad SYHG which was related to the P-pathotype. Only 3.13% of the virus showed a new tetrad AYPR which was related by phylogenicity to the A-pathotype of the RNA3 (Table 4.4).

Table 4.4. Comparison of sequences of portions of RNA3 of BNYVV isolates from several parts of the world and other BNYVV pathotypes.

Source type	Accession N°	Origin	Percentage occurrence in French BNYVV	Percentage occurrence in Iranian BNYVV	Amino acid position number																				
					213	238	230	322	322	449	667	668	669	670	738	1189	1292	1323	1379	1892	1983	2119			
A types																									
AHHG	AM745439-AM745500	Iran	/	27%	T	Y	I	S	H	L	T	A	H	H	G	C	N	H	L	P	N	V	T	D	D
AHHG	AY772230	Turquie 1	/	/	T	Y	I	S	H	L	T	A	H	H	G	C	N	H	T	P	D	V	T	D	D
AHHG	AY772235-AY772231	Turquie 8 and 24	/	/	T	Y	I	S	H	L	T	A	H	H	G	C	N	H	L	P	D	V	T	D	D
AHHG	AY734499	France	1,14%	/	T	T	I	S	H	L	T	A	H	H	G	C	N	H	L	P	N	V	T	D	D
AHHG		Sweden	/	/	T	Y	I	S	?	L	T	A	H	H	G	C	?	H	L	P	?	?	T	D	D
ACHG	AM745501-AM745558	Iran	/	27,10%	T	Y	I	S	H	L	T	A	C	H	G	C	N	H	L	P	N	V	T	D	D
ACHG	FM210566-FM210568	Iran	/	0,90%	T	Y	I	S	H	L	T	A	C	H	G	R	N	H	L	P	N	V	T	D	D
ACHG	AY696170	Spain	/	/	T	Y	I	S	H	L	T	A	C	H	G	C	N	H	L	P	N	V	T	D	D
ACHG	AY772232-AY772234-AY772233	Turquie 9 adn 11 and 19	/	/	T	Y	I	S	H	L	T	A	C	H	G	C	N	H	L	P	D	V	T	D	D
AYHG	AM745559-AM745596	Iran	/	23%	T	Y	T	S	H	L	T	A	Y	H	G	C	N	H	L	P	N	V	T	D	D
AYHG	AY696163	Japan	/	/	T	Y	I	S	H	L	T	A	Y	H	G	C	N	H	L	P	D	V	T	D	D
AYHG	AJ239200	China	/	/	T	Y	S	S	H	L	T	A	Y	H	G	C	N	N	I	S	N	V	T	D	D
AYPR	This study	France	3,13%	/	T	Y	I	S	H	S	T	A	Y	P	R	C	N	H	L	P	D	V	T	D	D
ALHG	AM745597-AM745615	Iran	/	9%	T	Y	I	S	H	L	T	A	L	H	G	C	N	H	L	P	N	V	T	D	D
ALHG	AF197553	Kazakhstan	/	/	T	Y	I	S	H	L	T	A	L	H	G	C	N	H	L	P	N	V	T	G	G
ALHG	AF197545	France (P type)	1,14%	/	T	Y	I	S	H	V	T	A	L	H	G	C	N	H	L	P	N	V	T	G	G
ALHG	AF197549	France (P type)		/	T	Y	I	S	H	L	T	A	L	H	G	C	N	H	L	P	N	V	T	G	G
ALHG	AF197558	Netherlands	/	/	T	Y	I	S	H	L	T	A	L	H	G	C	N	H	L	P	N	V	I	D	G
ALHG	AF197551	Italy	/	/	T	Y	I	S	H	L	T	A	L	H	G	C	N	H	L	P	N	V	T	D	D
ALHG	AY696127	USA	/	/	T	Y	I	S	H	L	T	A	L	H	G	C	N	H	L	P	N	V	T	D	D
AFHR	AM745637-AM745636	Iran	/	2,30%	T	Y	I	S	H	S	T	A	F	H	R	C	N	H	L	P	N	V	T	D	D
AFHR	AY696137	France	1,42%	/	T	Y	I	S	H	S	T	A	F	H	R	C	N	H	L	P	N	V	T	D	D
AFHR	AY696164	Netherlands	/	/	T	Y	I	S	H	S	T	A	F	H	R	C	N	H	L	P	N	V	T	D	D
AFHG	AM745637-AM745638	Iran	/	17%	T	Y	I	S	H	L	T	A	F	H	G	C	N	H	L	P	N	V	T	D	D
AFHG	AY696126	Austria	/	/	T	Y	I	S	H	L	T	A	F	H	G	C	N	H	L	P	N	V	T	D	D
AFHG	AY823275	Spain	/	/	T	Y	I	S	H	L	T	A	F	H	G	C	N	H	L	P	N	V	I	D	D
AFHG	AB207805	Japan	/	/	T	Y	I	S	H	L	T	A	F	H	G	C	N	N	I	S	N	V	A	D	D
AHYG	FJ230957	Iran	/	0,33%	A	Y	I	S	H	L	T	A	H	Y	G	C	N	H	L	P	N	V	T	D	D
VLHG	FM210618	Iran	/	0,33%	T	Y	I	S	H	L	T	V	L	H	G	C	N	H	L	P	N	V	T	D	D
VHHG	FJ230958	Iran	/	0,33%	T	Y	I	S	H	L	T	V	H	H	G	C	N	H	L	P	N	V	T	D	D
VCHG	This study (Garden beet)	France	0,28%	/	T	Y	I	S	H	L	T	V	C	H	G	C	?	?	?	?	?	?	?	?	?
VCHG	This study (Sugar beet)	France	/	/	T	Y	I	S	H	S	T	V	C	H	G	C	N	H	L	P	N	V	T	D	?
P types																									
SYHG	AM745616-AM745630	Iran	/	8%	T	Y	T	C	H	L	S	S	Y	H	G	C	N	N	I	S	N	V	A	D	D
SYHG	DQ462124	Kazakhstan	/	/	T	Y	T	R	H	L	S	S	Y	H	G	C	N	N	I	S	N	V	A	D	D
SYHG	AY696166-AY696167	France	/	/	T	Y	T	S	H	L	S	S	Y	H	G	C	N	N	I	S	N	V	A	D	D
SYHG	DQ440512	UK	/	/	T	Y	T	S	H	L	S	S	Y	H	G	C	N	N	I	S	N	V	A	D	D
SYHG	DQ415512-DQ415513	USA, IV	/	/	T	C	T	S	H	L	S	S	Y	H	G	C	N	N	I	S	N	V	A	D	D
SYHG	This study	France	19,77%	/	T	C	T	S	H	L	S	S	Y	H	G	C	N	N	I	S	N	V	A	D	D
SYHG	This study	France	0,33%	24,79%	T	Y	T	S	H	L	S	S	Y	H	G	C	N	N	I	S	N	V	A	D	D
SYHG	This study	France	0,66%	/	T	Y	P	S	H	L	S	S	Y	H	G	C	N	N	I	S	N	V	A	D	D
SYHG	This study	France	3,01%	/	M	Y	T	S	H	L	S	S	Y	H	G	C	N	N	I	S	N	V	A	D	D
B types																									
AYHR	AY696136	France	/	/	T	Y	I	S	N	L	S	A	Y	H	R	C	T	D	I	P	D	I	T	D	D
AYHR	This study	France	21,14%	70,37%	T	Y	I	S	N	L	S	A	Y	H	R	C	T	D	I	P	D	I	T	D	D
AYHR	This study	France	49,23%	/	M	Y	I	S	N	L	S	A	Y	H	R	C	T	D	I	P	D	I	T	D	D
TYHR	This study	France	0,85%	/	M	Y	I	S	N	L	S	T	Y	H	R	C	T	D	I	P	D	I	T	D	D

4.16. Is there an evolution of the BNYVV B-and P-pathotype content in the sugar roots from the field in a beet season?

A priori, the ELISA value linked to the AYHR (less multiplied in the plant) tetrad droped during the sugar beet season with *Rz1* cultivar and *Rz1Rz2* cultivar while the ELISA value linked to SYHG (well multiplied in the plant) increased. However, when the RNA3 content was looked (Figure 4.28), there was mainly an increase during the season, except for two varieties where there was a stability. In the first case, the RNA3 content linked with the tetrad AYHR (B-pathotype) was constant but the CP content decreased. In the second case, the RNA3 content linked with the tetrad AYHR increased and the CP content also. In the third, fourth, fifth and sixth cases, the RNA3 content linked here with the tetrad SYHG (P-pathotype) increased and the CP content increased also. In the last case, the RNA3 content linked with the tetrad SYHG was constant but the CP content increased. The tetrad SYHG influenced the RNA3 content and the CP content in the different varieties. We must keep in mind also that we were in three different soil contexts. We had the same soil context with three different varieties only in the three last cases.

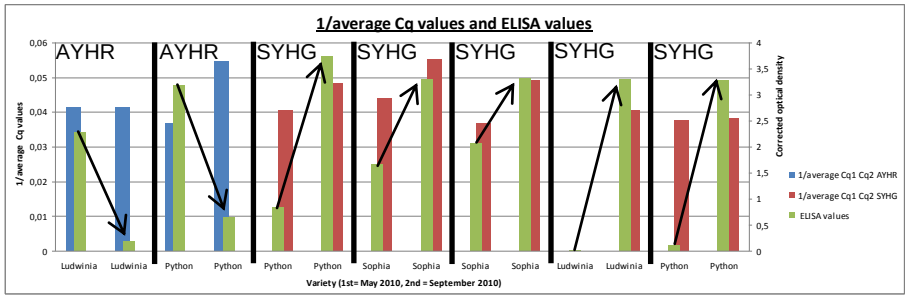


Fig. 4.28. 1/Cqnorm values of SYHG and AYHR tetrads, and the corrected optical density (ELISA values) as a function of different sugar beet varieties from 14 samples from fields 2010. Two adjacent histograms represent each time the values found for the young sugar beets in May (in left) and the older sugar beets in September (in right) from a same field. The first couple variety “Ludwina” and “Python” come from AI 2010. The second couple variety “Python” and the first couple variety “Sophia” come from AL 2010. The second couple variety “Sophia”, the second couple variety “Ludwina” and the third couple variety “Python” come from BL 2010.

4.17. In case of tetrad mixed infection in a single sugar root, what is the proportion of each tetrad in the sample?

In the seven samples analyzed here by RT-mqPCR and ELISA, the tetrad SYHG and AYHR were found by direct sequencing in double infection in each sample. With the direct sequencing, however, we had no real idea of the level of each tetrad in each sample-variety. Was there an effect of the variety on the level of certain tetrad? Was there an influence of a tetrad on the level of the other tetrad in case of mixed tetrad infection? The figure 4.29 shows the tetrads proportion in double infection (SYHG and AYHR) in a single root from field in September 2010. The ELISA values for all these samples were quite low, between 0,1 and 0,4. For five samples, the Rq was

higher for SYHG than for AYHR, between 2 and 33 times more concentrated. For the sample of Sophia in Gironville, Rq was 7 times higher for AYHR than for SYHG. For the sample of Ludwina in Chenou, Rq was 1,6 times higher for AYHR than for SYHG. In case of mixed infection (according to the few samples analyzed), the tetrad SYHG, in general, wa more concentrated that the AYHR tetrad.

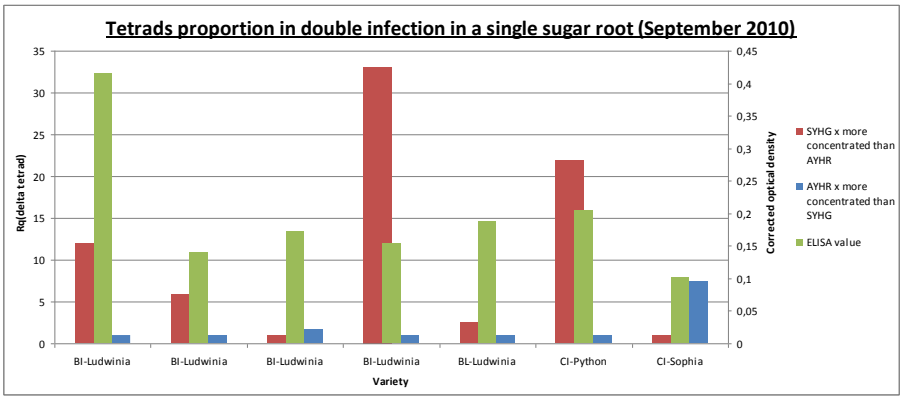


Fig. 5.29. Proportional relation between tetrads in double infection (AYHR and SYHG) in a single sugar root of three varieties from the field. ELISA values(corrected OD) and relative quantity (RT-mqPCR) of seven samples in September 2010. The Rq values represent the scaling factor between each tetrad, i.e. how much each tetrad is more concentrated compared to the other tetrad in a single sug ar root. There are four varieties 'Ludwina' from the field of Chenou, on 'Ludwina' from Yèvres-la-Ville, one 'Python' and one 'Sophia' from Gironville.

4.18. Is there a selection pressure of the different varieties used on the BNYVV diversity and the presence of associated viruses or a natural inter-field diversity?

The figure 4.30 represents the tetrads obtained by direct sequencing in the different varieties analyzed (844 samples) from the fields between 2008 and 2011. The varieties which showed the highest tetrad diversity were in order of importance Ludwina, Julietta, Britta, Python/Bison, Sophia/Magellan, Bering/Annouchka, Rosalinda, Baobab/Adriana and Fiorenza. Ludwina, Britta, Python, Sophia, Rosalinda, Magellan were implemented in field without nematode. The other varieties were implemented in field infected with nematodes and these varieties were resistant to *Heterodera schachtii*. In the assays, there were three different varieties implemented. In field that were almost homogeneous in tetrad for two varieties, the only variety which showed diversity was Ludwina. The variety with *Rz1* and *Rz2* genes applied a selection pressure on the virus or the double resistant varieties allowed a higher diversity of tetrads in sugar beet. These varieties, however, were less affected by symptoms than the others.

The figure 4.31.A, 4.31.B and 4.31.C represents the results for each field analysed in 2009, 2010 and 2011 respectively. These figures allowed to observe the correlation in the tetrad results between each variety for each field. In this way, we can observe the behaviour of three varieties per field.

The period of sampling had a important impact in the results. According to the period, the results were different and evolved. A viral evolution in time through the field was observed during the crop season. In 2010, this observation was very obvious for all the fields. There was almost no infection in May 2010 except for the two fields where the P-pathotype was detected. Indeed, these two fields showed a higher presence of the BNYVV in May than the other fields. And in September, these two fields were completely infected. In 2011, the difference between May and September was slight. The BNYVV was detected in each zone of each field in May 2011 except for the field from Teillay-le-Gaudin where there was less infection. In September 2011, the presence of BNYVV was slightly higher. The results were consequently also different in regard with the year investigated and the weather.

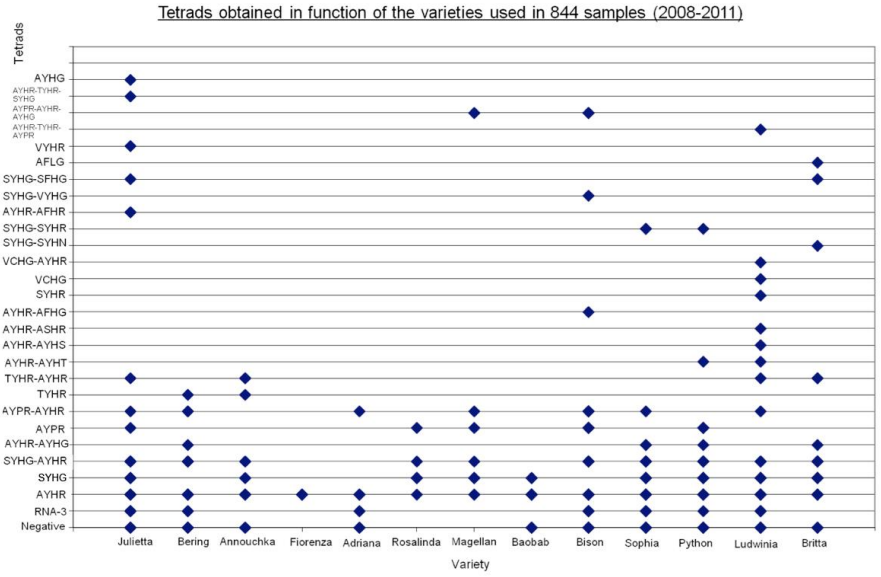
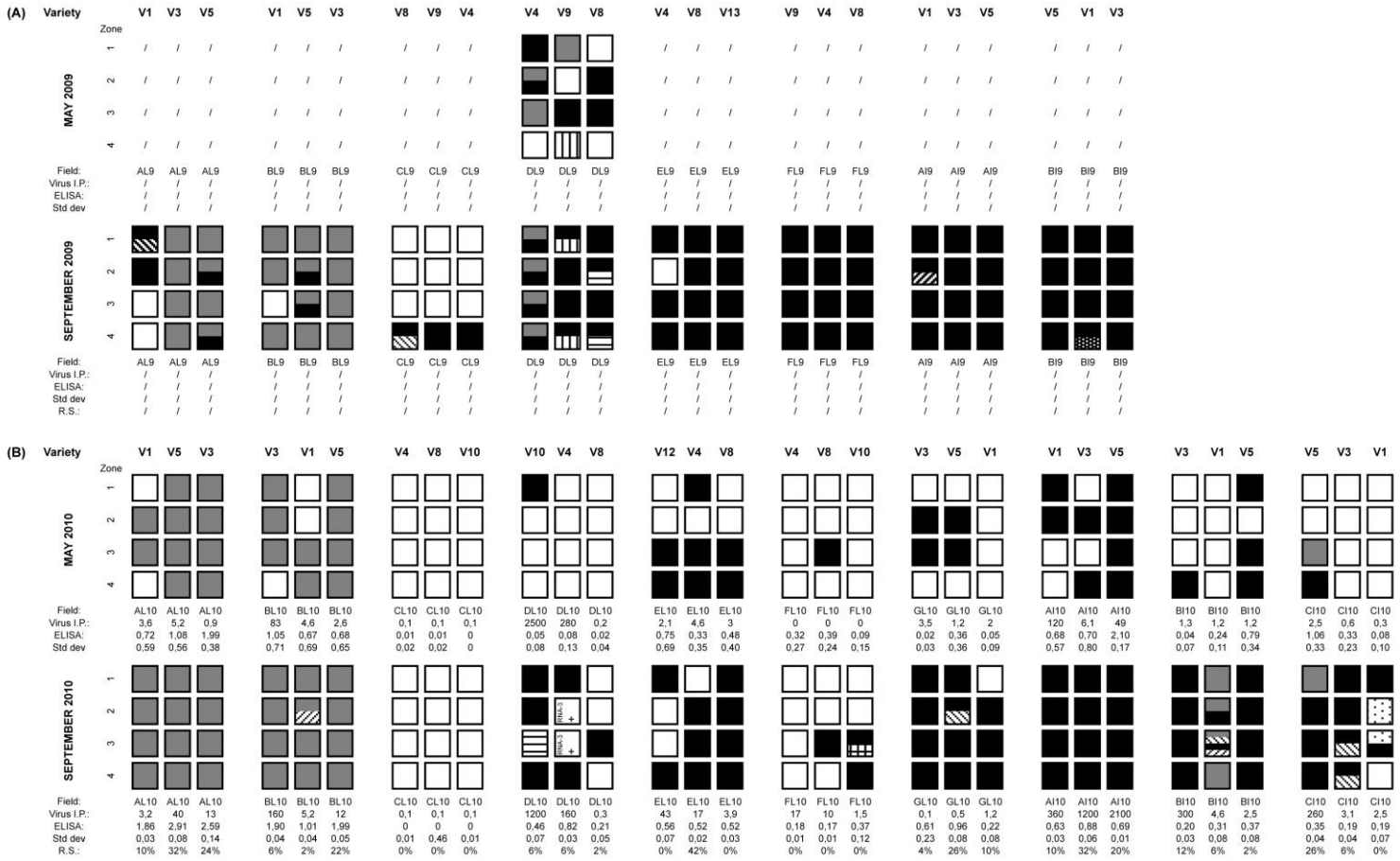


Fig. 4.30. Tetrads obtained by direct sequencing in the different varieties analyzed (844 samples) from the fields between 2008 and 2011. Among the 844 samples, there were 185 'Julietta', 67 'Bering', 21 'Annouchka', four 'Fiorenza', 12 'Adriana', 11 'Rosalinda', 16 'Magellan', 32 'Baobab', 112 'Bison', 116 'Sophia', 123 'Python', 93 'Ludwina' and 52 'Britta'. These proportions must be kept in mind when the different varieties are compared. There were 11 different tetrads or tetrad associations found in the variety 'Julietta', five in 'Bering', five in 'Annouchka', one in 'Fiorenza', two in 'Adriana', four in 'Rosalinda', six in 'Magellan', two in 'Baobab', seven in 'Bison', six in 'Sophia', seven in 'Python', 12 in 'Ludwina' and eight in 'Britta'.


(A)
Variety
V1 V3 V5
V1 V5 V3
V8 V9 V4
V4 V9 V8
V4 V8 V13
V9 V4 V8
V1 V3 V5
V5 V1 V3
Zone
MAY 2009
Field:
Virus I.P.:
ELISA:
Std dev
AL9 AL9 AL9
BL9 BL9 BL9
CL9 CL9 CL9
DL9 DL9 DL9
EL9 EL9 EL9
FL9 FL9 FL9
AI9 AI9 AI9
BI9 BI9 BI9
SEPTEMBER 2009
R.S.:
(B)
Variety
V1 V5 V3
V3 V1 V5
V4 V8 V10
V10 V4 V8
V12 V4 V8
V4 V8 V10
V3 V5 V1
V1 V3 V5
V3 V1 V5
V5 V3 V1
MAY 2010
Field:
Virus I.P.:
ELISA:
Std dev
AL10 AL10 AL10
3,6 5,2 0,9
0,72 1,08 1,99
0,59 0,56 0,38
BL10 BL10 BL10
83 4,6 2,6
1,05 0,67 0,68
0,71 0,69 0,65
CL10 CL10 CL10
0,1 0,1 0,1
0,01 0,01 0
0,02 0,02 0
DL10 DL10 DL10
2500 280 0,2
0,05 0,08 0,02
0,08 0,13 0,04
EL10 EL10 EL10
2,1 4,6 3
0,75 0,33 0,48
0,69 0,35 0,40
FL10 FL10 FL10
0 0 0
0,32 0,39 0,09
0,27 0,24 0,15
GL10 GL10 GL10
3,5 1,2 2
0,02 0,36 0,05
0,03 0,36 0,09
AI10 AI10 AI10
120 6,1 49
0,68 0,70 2,10
0,57 0,80 0,17
BI10 BI10 BI10
1,3 1,2 1,2
0,04 0,24 0,79
0,07 0,11 0,34
CI10 CI10 CI10
2,5 0,6 0,3
1,06 0,33 0,08
0,33 0,23 0,10
SEPTEMBER 2010
R.S.
AL10 AL10 AL10
3,2 40 13
1,86 2,91 2,59
0,03 0,08 0,14
10% 32% 24%
BL10 BL10 BL10
160 5,2 12
1,90 1,01 1,99
0,04 0,04 0,05
6% 2% 22%
CL10 CL10 CL10
0,1 0,1 0,1
0 0 0
0,01 0,46 0,01
0% 0% 0%
DL10 DL10 DL10
1200 160 0,3
0,46 0,82 0,21
0,07 0,03 0,05
6% 6% 2%
EL10 EL10 EL10
43 17 3,9
0,56 0,52 0,52
0,07 0,02 0,03
0% 42% 0%
FL10 FL10 FL10
17 10 1,5
0,18 0,17 0,37
0,01 0,01 0,12
0% 0% 0%
GL10 GL10 GL10
0,1 0,5 1,2
0,61 0,96 0,22
0,23 0,08 0,08
4% 26% 10%
AI10 AI10 AI10
360 1200 2100
0,63 0,88 0,69
0,03 0,06 0,01
10% 32% 20%
BI10 BI10 BI10
300 4,6 2,5
0,20 0,31 0,37
0,03 0,08 0,08
12% 6% 2%
CI10 CI10 CI10
260 3,1 2,5
0,35 0,19 0,19
0,04 0,04 0,07
26% 6% 0%

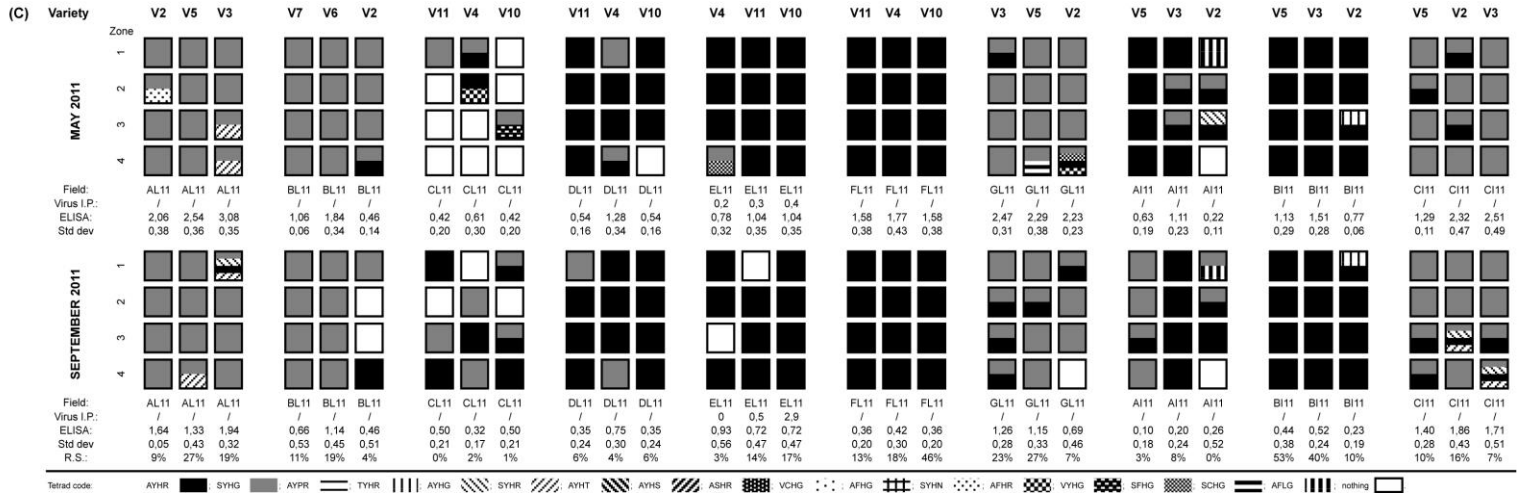


Fig. 4.31. Representation of the tetrad diversity for three varieties in each field (12 zones) for the period of the beginning of the culture (May) and the end of the culture (September) in the region of Pithiviers (France). A. Fields of 2009. B. Fields of 2010. C. Fields of 2011. For each band, the virus MPN, the root severity (RS) and the average ELISA values are indicated. The code of the varieties are the following: V1, Ludwina; V2, Britta; V3, Sophia; V4, Julietta; V5, Python; V6, Magellan; V7, Rosalinda; V8, Béring; V9, Annouchka; V10, Bison; V11, Baobab; V12, Adriana; V13, Fiorenza.

The figure 4.32 shows the percentage occurrence of RNA3 in each variety analyzed among 843 samples between 2008 and 2011. The BNYVV RNA3 was highly present in the different varieties analyzed. Overall, the varieties were infected between 60% and 100% by the RNA3 of the BNYVV. Proportionally of the number of each variety compared of all varieties analyzed, the variety Julietta was the most infected followed by Python, Sophia, Bison, Ludwina, Britta and Bering.

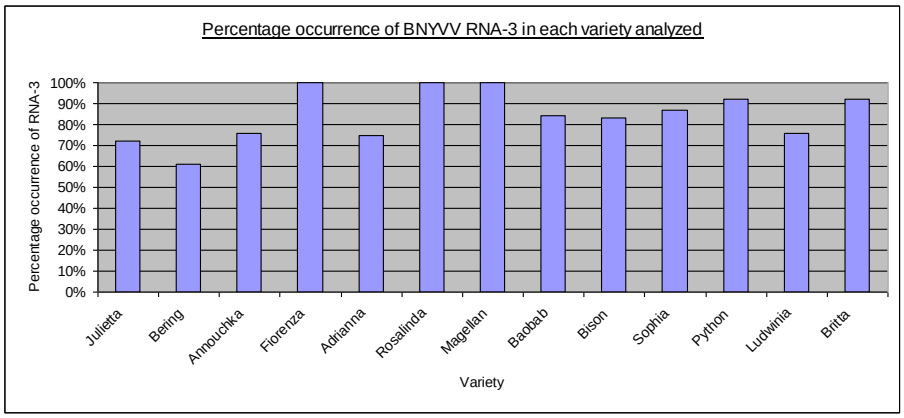


Fig. 4.32. Percentage occurrence of RNA3 in each variety analyzed among 843 samples between 2008 and 2011. Among the 843 samples, there were 185 'Julietta', 67 'Bering', 21 'Annouchka', four 'Fiorenza', 12 'Adriana', 11 'Rosalinda', 16 'Magellan', 32 'Baobab', 112 'Bison', 116 'Sophia', 123 'Python', 92 'Ludwina' and 52 'Britta'. These proportions must be kept in mind when the different varieties are compared.

If we proportionally (weighting factor) compared the different varieties with regard to their infection by the fifth RNA, the variety Julietta was still the most infected by BNYVV RNA5 followed by Bison/Python/Sophia, Britta, Ludwina, Baobab, Magellan/Bering and Annouchka/Rosalinda (Figure 4.33). We must not forget that these different varieties were implemented in different fields where there were different BNYVV diversities. The different fields were heterogeneous between them.

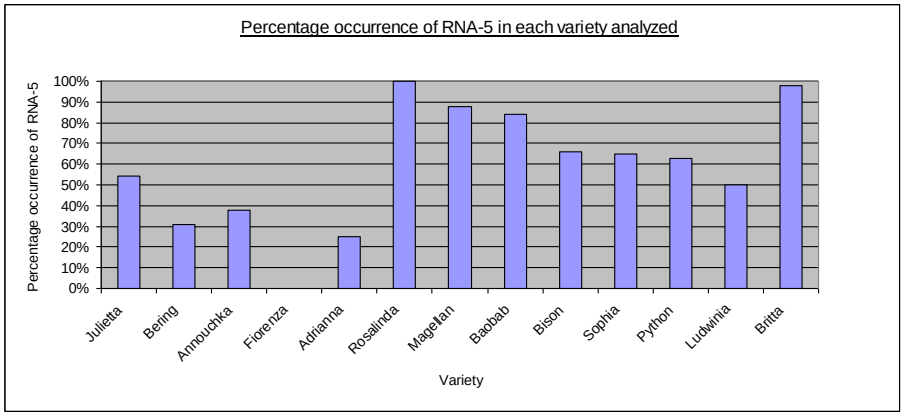


Fig. 4.33. Percentage occurrence of RNA5 in each variety analyzed among 843 samples between 2008 and 2011. Among the 843 samples, there were 185 'Julietta', 67 'Bering', 21 'Annouchka', four 'Fiorenza', 12 'Adriana', 11 'Rosalinda', 16 'Magellan', 32 'Baobab', 112 'Bison', 116 'Sophia', 123 'Python', 92 'Ludwina' and 52 'Britta'. These proportions must be kept in mind when the different varieties are compared.

The figure 4.34 shows the percentage occurrence of RNA2 in each variety analyzed among 705 samples between 2008 and 2011. If we proportionally (weighting factor) compared the different varieties with regard to their infection by the second RNA, the variety Python was the most infected by BNYVV RNA2 followed by Sophia, Julietta/Bison, Ludwina, Baobab and Britta/Bering. Overall, the RNA2 was less detected than the RNA3. This could be due to the degradation by the resistance of the sugar beet of the RNA2 which comprises the PTGS inhibitor.

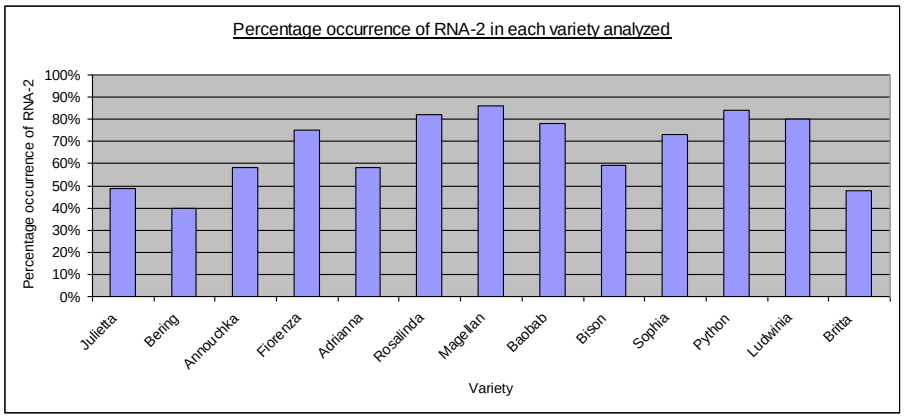


Fig. 4.34. Percentage occurrence of RNA2 in each variety analyzed among 705 samples between 2008 and 2011. Among the 705 samples, there were 136 'Julietta', 48 'Bering', 12 'Annouchka', four 'Fiorenza', 12 'Adriana', 11 'Rosalinda', 14 'Magellan', 32 'Baobab', 112 'Bison', 104 'Sophia', 104 'Python', 66 'Ludwina' and 50 'Britta'. These proportions must be kept in mind when the different varieties are compared.

The figure 4.35 and 4.36 shows the percentage occurrence of respectively BSBV and BVQ in each variety analyzed among 743 samples between 2008 and 2011. BVQ was clearly must present than BNYVV in general and infected all the varieties between 70% and 100%. The figure 4.35 shows clearly the effect of the double resistance varieties (*Rz1rz1Rz2rz2*) on the presence of BSBV. There was an inhibition of this virus with these varieties and moreover the observed symptoms for the double resistance varieties were less aggressive. Nevertheless, BSBV was quite present in the other *Rz1rz1* varieties analyzed.

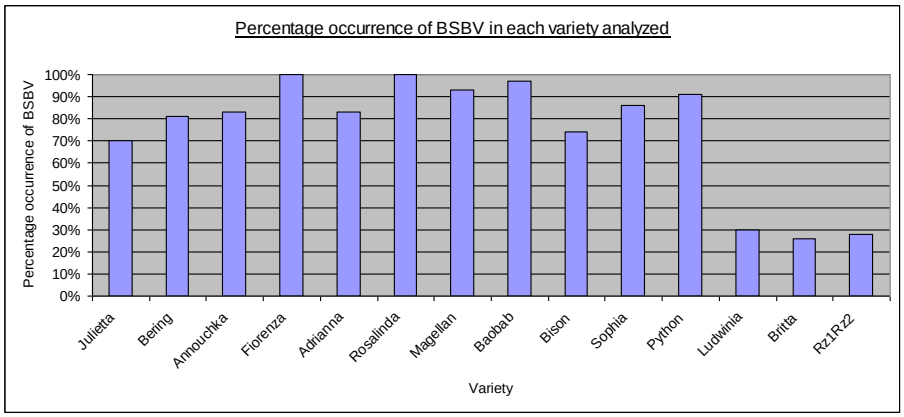


Fig. 4.35. Percentage occurrence of BSBV in each variety analyzed among 743 samples between 2008 and 2011. Among the 743 samples, there were 170 'Julietta', 48 'Bering', 12 'Annouchka', four 'Fiorenza', 12 'Adriana', 11 'Rosalinda', 14 'Magellan', 32 'Baobab', 112 'Bison', 104 'Sophia', 108 'Python', 66 'Ludwina' and 50 'Britta'. The result for all the *Rz1rz1Rz2rz2* varieties (116 samples) is also shown here. These proportions must be kept in mind when the different varieties are compared.

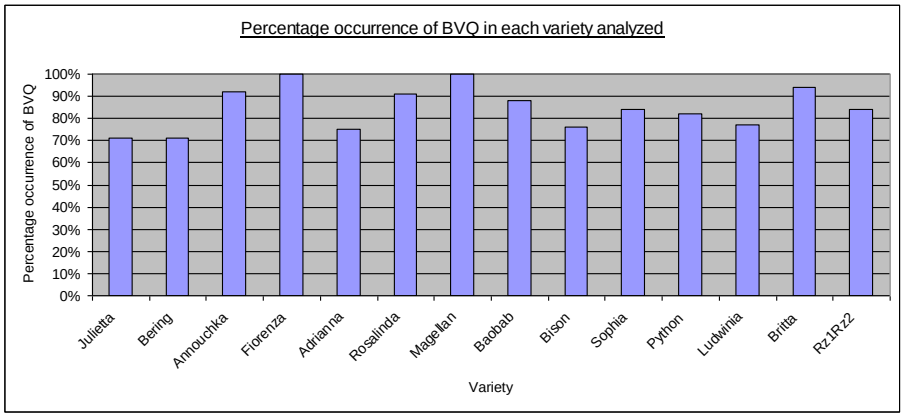


Fig. 4.36. Percentage occurrence of BVQ in each variety analyzed among 743 samples between 2008 and 2011. Among the 743 samples, there were 170 'Julietta', 48 'Bering', 12 'Annouchka', four 'Fiorenza', 12 'Adriana', 11 'Rosalinda', 14 'Magellan', 32 'Baobab', 112 'Bison', 104 'Sophia', 108 'Python', 66 'Ludwina' and 50 'Britta'. The result for all the *Rz1rz1Rz2rz2* varieties (116 samples) is also shown here. These proportions must be kept in mind when the different varieties are compared.

4.19. Is it due to something else in the virus level which is not put in evidence with our indicators?

The rhizomania problematic was a multifactorial complex. During our research, the p25, p26 and p75 was mainly detected in the BNYVV. But, the protein p31 coded by the RNA4 was also known to act in the pathogenicity. Unfortunately, we didn't have the possibility to test this protein during our research. Maybe, this protein was also a part of the complex answer. Moreover, the presence of RNA2 was detected for the protein p75 by the multiplex RT-qPCR but the real protein acting with the plant was the p14

which was a PTGS suppressor. The different RNAs of the BNYVV were also less present when the double resistance varieties were put in place. Could this double resistance act also in preventing the PTGS suppressing?

4.20. Is it due to an external parameter as virus-nematode synergism or associated viruses?

It was evidenced that the double resistance varieties as Ludwina and Britta (*Rz1rz1Rz2rz2*) had the possibility to control the presence of BSBV. With this kind of varieties there were also less infection and less root severity in the field. However, the BNYVV tetrad diversity was higher in theses two varieties compared to other varieties which possessed only the major resistance *Rz1rz1*. The double resistance varieties were only used in field no-infested by nematode. Theses two varieties didn't possess the resistance against nematode.

4.21. Must we lengthen the rotation period in order to decrease the infectious potential?

The big resistance-breaking events that occurred in the region of Pithiviers showed that a culture of beet every three years seems still not decrease the resistance-breaking events. Moreover, irrigation worsens the disease in favouring the infectious potential. In France, viral MPN (infectious potential) can achieve in certain varietal band 2100 IU/g of soil or per plot, 680 IU/g of soil. With regard to the vector MPN, the values were often higher than 23 000 IU/g of soil. Comparing to the results in Belgium of Goffart and Maraite (1992), the MPN in France were very huge! The figures below (Figure 4.37) showed that the tetrad AYHR linked to the B-pathotype was preferentially linked that the tetrad SYHG to higher viral and vector MPN. That's why the B-pathotype was the most encountered in the region of Phitiviers before the P-pathotype. This figure explained consequently that the huge dispersion of the B-pathotype in France was probably due to its good relation with the vector.

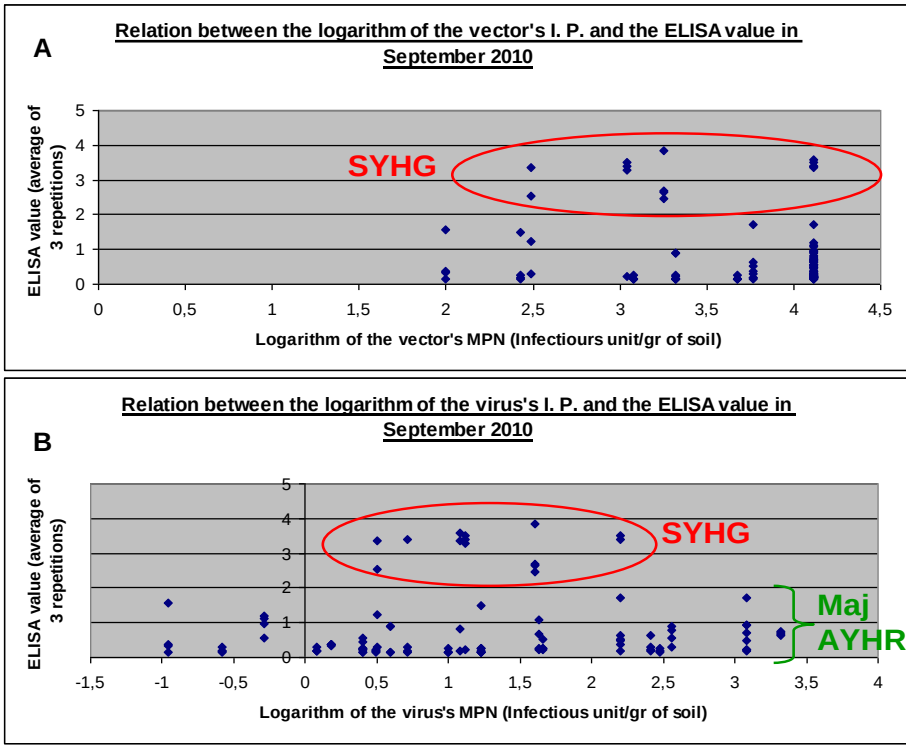


Fig. 4.37. Relation between the logarithm of the vector MPN (infectious potential) (A) or virus MPN (infectious potential) (B) and the ELISA values (corrected OD) of 120 samples in September 2010.

There were no relations between the ELISA values observed and the virus or vector MPN (Figure 4.38). But, this figure shows well the higer level of vector MPN compared to virus MPN.

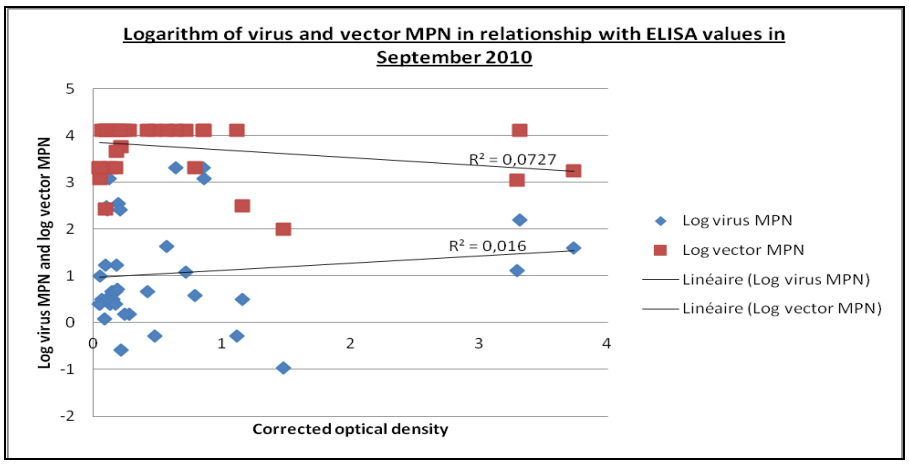


Fig. 4.38. Relation between the logarithm of the vector MPN and virus MPN and the ELISA (corrected OD) values of 38 samples in September 2010.

There were no strict correlation between the logarithm of virus MPN or vector MPN and the root severity. The root severity was only explained for 11,83% by the virus MPN (Figure 4.39).

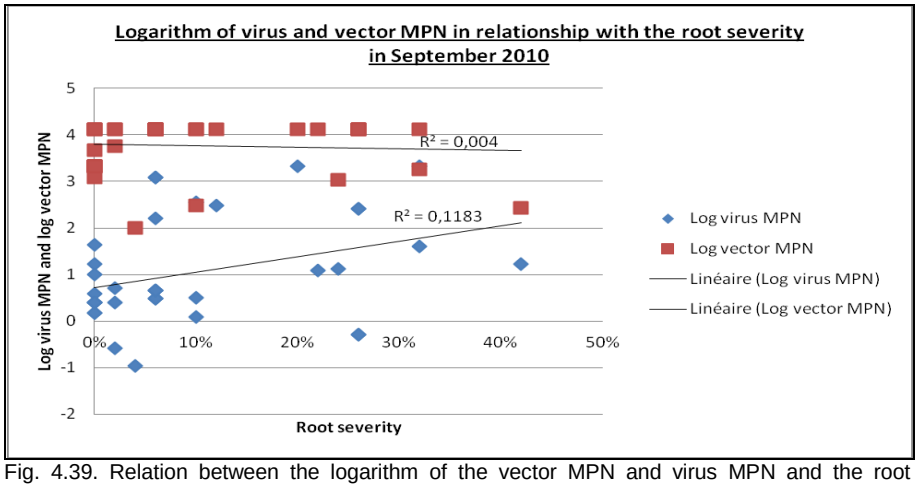


Fig. 4.39. Relation between the logarithm of the vector MPN and virus MPN and the root severity of 38 samples in September 2010.

In the figure 4.40, the results came from the varieties used in the ten fields implemented in September 2010. There were 38 samples selected for the analyses by RT-mqPCR. In the different varieties, there were simple infection of each pathotype and also double infection of pathotype. The correlation between the RT-mqPCR results and the vector MPN was strong. For the tetrad SYHG, the level of this tetrad in the sugar beet root was explained for 97,99% by the level of vector MPN. The relative quantity of the tetrad SYHG in the sugar beet root decreased strongly when the vector MPN increased. It was, however, the opposite for the tetrad AYHR. The level of this tetrad in the sugar beet root was explained for 32,93% by the level of vector MPN. When the vector MPN increased, the level of AYHR in the sugar beet root increased also. Unfortunately, a vector MPN detection threshold was reached which did not allow us to know the precise value of the highest MPN. Consequently, the correlation coefficient should be higher for AYHR. The tetrad AYHR was in good relation with its vector and that was why the majority of the roots were mainly infected by the tetrad AYHR. However, the tetrad SYHG was in bad relation with its vector and the high level of SYHG in some roots was not due to the vector but due to the high multiplication level of this tetrad in the sugar beet root. Consequently, some vector contained sometimes the tetrad SYHG and when the vector infected the roots, the tetrad SYHG would compensate for its poor relationship with its vector showing a better ability to multiply itself in the taproot.

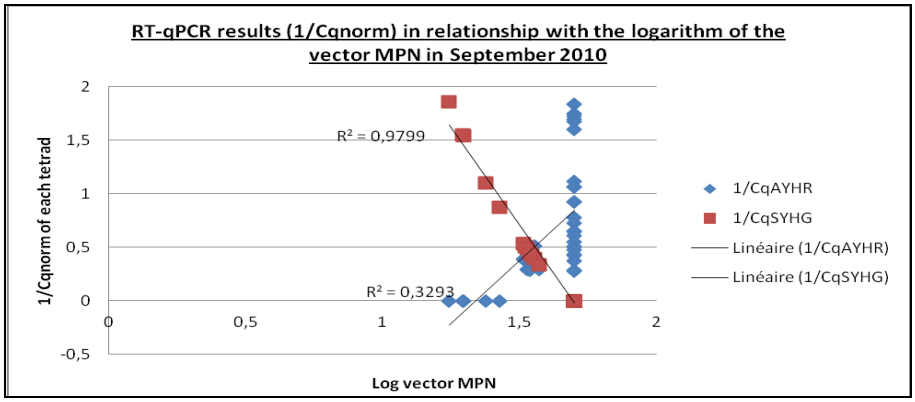


Fig. 4.40. Relation between the logarithm of the vector MPN and the RT-mqPCR results (1/Cqnorm) of 38 samples in September 2010.

The correlation between the RT-mqPCR results and the virus MPN (viruliferous *Polymyxa betae*) was strong but differently than the vector MPN (Figure 4.41). For the tetrad AYHR, the level of this tetrad in the sugar beet root was explained for 97,37% by the level of virus MPN. The relative quantity of the tetrad AYHR in the sugar beet root decreased strongly when the virus MPN increased. It is, however, the opposite for the tetrad SYHG. The level of this tetrad in the sugar beet root was explained for 40,48% by the level of virus MPN. When the virus MPN increased, the level of SYHG in the sugar beet root increased also. Unfortunately, a virus MPN detection threshold was reached which did not allow us to know the precise value of the highest MPN. Consequently, the correlation coefficient would be higher for SYHG. This explains that the tetrad SYHG infected also the plant through the vector. And it was more important for the tetrad SYHG than the tetrad AYHR to have a virus MPN high. This was because the tetrad SYHG had a poor relationship with its vector and it was important for this tetrad to have enough relationship to have the possibility to infect the sugar beet root and to multiply after inside.

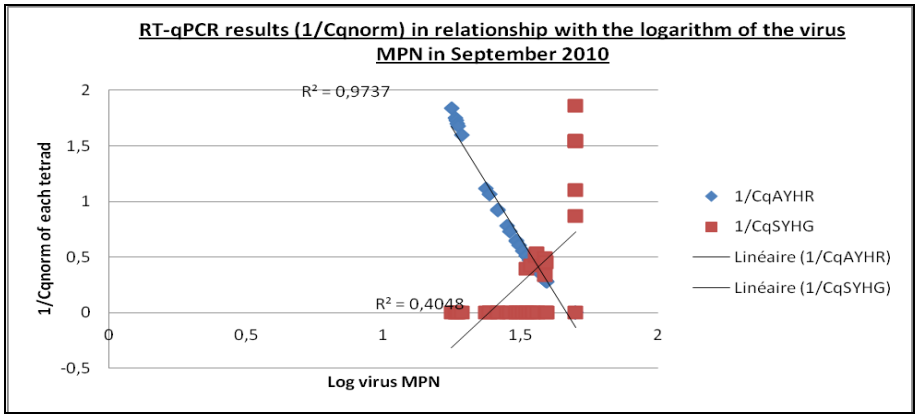


Fig. 4.41. Relation between the logarithm of the virus MPN and the RT-mqPCR results (1/Cqnorm) of 38 samples in September 2010.

4.22. What is the type of infestation in the entire root of some varieties?

To answer this question, section roots of the variety Bison (DL10) and the variety Julietta (DL10) were studied. These varieties came from the highly diversified field from Sougy (DL10) in September 2010. Leaves and six sections of the sugar beet taproot were analysed. The different sections showed different results. There was an heterogeneity in the infestation type between the different sections and between the different sugar beet varieties (Figure 4.42). There was a heterogeneity intra-field, intra-variety and inside a single sugar beet plant.

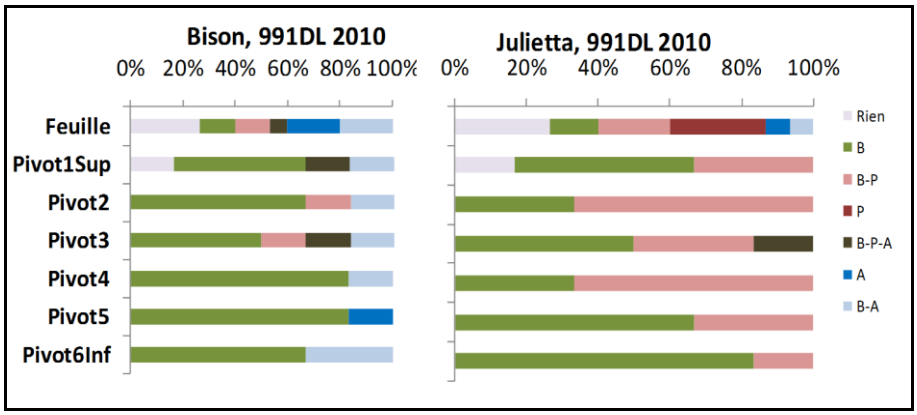


Fig. 4.42. Study of root sections in the variety Bison and Julietta from the same field (Sougy) in September 2010.

Mixed infection of pathotype (tetrad: SYHG, AYHR, AYPR, TYHR, AYHG) were observed with a larger proportion of the B-pathotype. BVQ was almost present in 100% of all the sections and was therefore homogeneously distributed in the taproot. There were heterogeneous distribution in the taproot for the fifth BNYVV RNA, the BNYVV RNA2 and BSBV. The P-pathotype RNA3 was rather upside in the taproot and in the leaves. The P-pathotype RNA5 was rather downside in the taproot.

4.23. Short conclusion

If we had had the possibility to know if there was a strict correlation between ELISA values and yield losses or between the root severity and yield losses, we could have had the possibility to link the yield losses with the exact causes of rhizomania in France. Precisely, if the yield losses were linked to the ELISA values, then we could have linked the yield losses to the presence of the SYHG. Indeed, we demonstrated that the tetrad SYHG was linked to high ELISA values compared to the tetrad AYHR. The tetrad SYHG allowed a better virus accumulation and multiplication inside the sugar beet root. A modulation in the production of each RNA as a function of the varietal context and according to the period was supposed. That's why the RT-mqPCR was not directly close of the ELISA values. But, there was a strict

correlation between the RT-mqPCR results of SYHG and the ELISA values. Moreover, there was also a strict correlation between the RT-mqPCR results of AYHR and the ELISA values. It was not the case between the RT-mqPCR results and the root severity. Consequently, there was a correlation between the tetrad-type coded by RNA3 and CP coded by RNA2. When AYHR content increased strongly, the CP content increased slightly. But, when SYHG content increased strongly, the CP content increased also strongly. When there was SYHG and AYHR mixed together, the CP content remained constant. The mixed tetrad infection influenced the level of SYHG in the sugar beet root and its multiplication.

The double resistance gene (*Rz1rz1Rz2rz2*) showed a better control of BSBV than BNYVV. In the variety with such a resistance, the root severities were also lower. The associated viruses of rhizomania played an important role in the disease in France. Moreover, the variety Britta showed almost 100% of the samples infected by the fifth BNYVV RNA while there were only 50% of the samples infected by this RNA in the variety Ludwina.

There was a double balance between virus-vector and virus-plant in the selection of the tetrad. The tetrad had an impact on the disease. In term of transmission and conservation in the soil, the tetrad AYHR was known to be better linked with its vector than the tetrad SYHG. The ELISA and IP results showed this as Diane Doucet observed it too. But, there was a comparative advantage between the tetrad SYHG and AYHR. The tetrad SYHG movement and multiplication was favored in the plant. Some vector contained sometimes the tetrad SYHG and when the vector infected the roots, the tetrad SYHG would compensate for its poor relationship with its vector showing a better ability to multiply itself in the taproot. It was, however, not the case for the tetrad AYHR. Consequently, if the rotation period increases, the presence and the level of the tetrad SYHG will decrease in the field and the sugar roots. It is because the MPN and the accumulation in the vector of the tetrad SYHG was lower than the tetrad AYHR.

CHAPTER 5
GENERAL DISCUSSION, CONCLUSION & PERSPECTIVES

GENERAL DISCUSSION

The main objective of this thesis was to provide a better understanding of the rhizomania epidemic in France, through a thorough evaluation of the spatio-temporal diversity of BNYVV at field level, based on a long-term approach. The rhizomania syndrome was the result of a complex interaction between a virus, BNYVV, a protozoan vector, *Polymyxa betae* and a host plant, *Beta vulgaris* was the heart of the rhizomania syndrome (Figure 5.1). The complexity of such disease came from the fact that it was linked to the multiple parameters, which taken one by one, would have no connection with the symptoms or the effect of the disease on yield. Field-based approaches must rely on such complexity, and it was one of the challenges of this thesis to deal with it through the generation of large data sets.

To address the scientific questions raised by the rhizomania disease, two different strategies were available: firstly, a top-down approach based on reverse genetics and the use of infectious full length clones of BNYVV of different pathotypes and secondly, a bottom-up approach based on field-based trials (Table 5.1). Unfortunately, until now, the construction of infectious clones of BNYVV was only successful for BNYVV B-pathotype (Quilley *et al.*, 1989). Therefore, the second approach was chosen.

Interfering parameters from the field approach added constraints. These parameters were either biotic or abiotic, like the frequent co-occurrence of other soil-borne viruses, nematodes (*Heterodera schachtii*)) or climatic variation, humidity, the different soils, the previous crop in the rotation, presence of other bacterial or fungal pathogens, …(Figure 5.1).

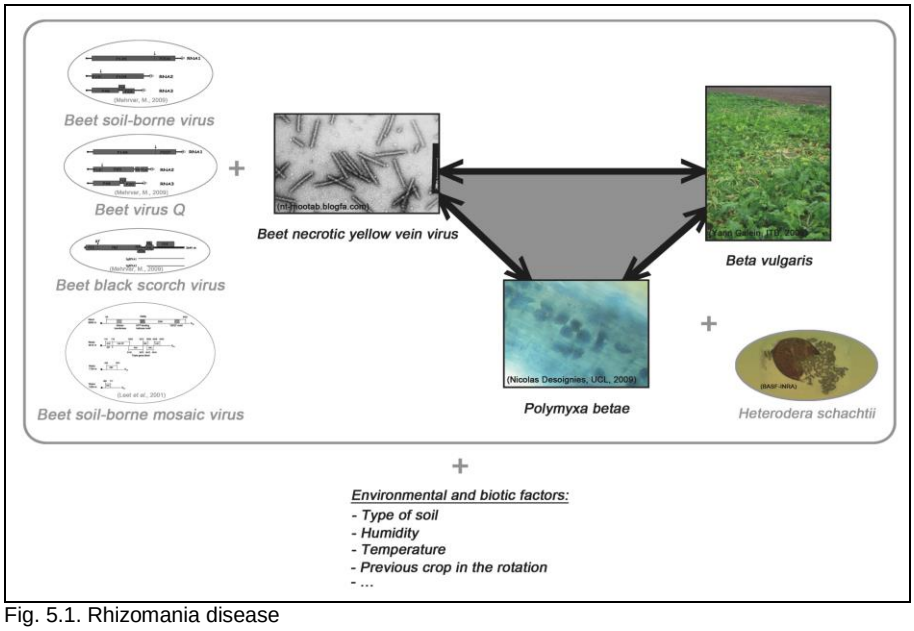


Fig. 5.1. Rhizomania disease

At the start of the analysis, technical questions raised with regard to the evaluation of the diversity of the virus and the vector at field level and sugar beet level. We observed an intra- and inter-plot diversity of the virus and the vector. There were also an intra- and inter-beet diversity. Indeed, we evidenced BNYVV pathotypes diversity and viruses diversity in the root samples. According to the position sampled in a single taproot, the results were different. We therefore opted for a representative and homogeneized system of analysis. It was not feasible to analyze systematically the entire root for each sample. Five to six root tips and rootlets in the same zone were taken

Table 5.1. Reverse genetics and field based approaches

Two possible strategies	
Reverse genetics approaches	**Field based approaches**
Discover the function of a gene by analyzing the phenotypic effects of specific gene sequences obtained by DNA sequencing	Seeks to find the genetic basis of a phenotype or trait
Advantages	**Advantages**
1) To learn the influence a sequence has on phenotype, or to discover its biological function, researchers can engineer a change or disruption in the genome	1) A researcher would locate (map) the gene on its chromosome by crossbreeding with individuals that carry other unusual traits and collecting statistics on how frequently the two traits are inherited together
2) Site-direct mutagenesis	2) Results in relationship with the reality of the field
3) Transgenic organism	3) Simple technique
Drawbacks	**Drawbacks**
1) Complex technology	1) Interaction with all parameters
2) Unsuccessful for BNYVV clones until now	2) Contamination, not controlled conditions

per sample. All the roots in each sample were pooled and homogeneized for the analysis to have the same repetitive results. We analyzed also single roots to the behavior of the virus and its diversity in one root. In the Pithiviers area, there were high variability in the tetrad of BNYVV and the three pathotypes of BNYVV were present in the area with a P-pathotype dispersion from Pithiviers. The rhizomania-infested soil from the upper 25 cm of each zone for each field was also analyzed and showed high infectious potentials. To evaluate the infection during a single sugar beet season, the studies of the evolution of the infectious potentials between the beginning of the culture and the end of the culture very useful. To evaluate the infection in the field assays and its dynamic, the end of the culture was the best period because a majority of the beet were infected at this period compared to the beginning. Different varieties were then compared in the same field and they showed different behaviours.

Both the virus and its vector should be mapped with strategic samples and appropriate technologies (Table 5.2). Well-known technologies were used in this thesis all together with newly developed ones.

Table 5.2. Technical questions.

Diversity of BNYVV/*Polymyxa betae* at field level and sugar beet level	
Technologies used:	
Most Probable Number of the virus and the vector in the soil	Goffart *et al.*, 1987, 1991 & 1992; Tuitert *et al.*, 1990 & 1994
DAS-ELISA methods for BNYVV quantitation	Torrance *et al.*, 1988; Koenig *et al.*, 1998
RT-PCR of *Polymyxa betae*	Legrève *et al.*, 2002
Multiplex RT-PCR of BNYVV, *P. betae*, BSBV & BVQ	Meunier *et al.*, 2003
BNYVV diversity and dispersion in France	Schirmer *et al.*, 2005, Nagy *et al.*, 2008
Whole sugar beet root lyophilisation and grinding for homogeneisation	This study
Study of section of sugar beet root for BNYVV, *P. betae*, BSBV & BVQ distribution	This study
Quantitative multiplex RT-PCR (Taqman Technology) of BNYVV RNA-3	This study
Deep-sequencing of RNA-2, RNA-3 & RNA-5 in different resistance background (selection pressure)	This study

The proposed thesis contributed to the current knowledge on the rhizomania disease (Table 5.3): this work contributed to evidence a wider diversity of the BNYVV in the Pithiviers area compared to the previous observations of Schirmer *et al.* (2005) and Nagy *et al.* (2008). For each sample, the presence of rhizomania associated viruses was tested as well as the presence of nematodes. The level of BNYVV in the sugar beet root was analyzed. And the link with the variety was always noticed. Several varieties with different resistance background were analyzed. The presence of BNYVV RNA2, RNA3 and RNA5 was tested for each sample. In patches

from farmer's field, we analyzed always a sick variety and a healthy variety to have the comparison in the results obtained.

Table 5.3. Contribution of the thesis to the current knowledge on rhizomania.

Contributions:	Paragraphs:
Variation in the sugar beet resistance and its expression	Rz1rz1 & Rz1rz1Rz2rz2 resistance
Comparison of three BNYVV type (A, B & P)	BNYVV identity stability
Explosion of the BNYVV tetrad diversity in the Pithiviers area	BNYVV tetrad diversity
Tetrads associated with resistance-breaking	P25 as Avr and virulent factor
Study of sections in a single sugar beet taproot	Compartimentalization of BNYVV type in sugar beet root
Mixed infection of BNYVV type and associated viruses in roots High level of BNYVV B-type followed by P-type in France High level of BNYVV and *P. betae* in the soil	Differences between France and USA
Increase and dispersion of the P-type in the Pithiviers area	BNYVV and dispersion of P-type to the East
High frequency of *Pomoviruses* associated in the Pithiviers area	*Pomoviruses* associated
Decrease of the root severity with Rz2 resistance gene	Resistance of Rz1rz1Rz2rz2 varieties to BSBV
Increase of the infectious potential of the soil during the season	High virus and vector MPN
Presence of BSBV, BNYVV and BVQ in a single sugar beet root	Inter genera viruses' competition
Mixed infection of AYHR and SYHG in a single sugar beet root with possible "reassortment"	Intra species virus' competition (reassortment or satellite?)
Effect of the variety on the BNYVV strains during the season	Microevolution and selection pressure

Resistance *Rz1rz1* and *Rz1rz1Rz2rz2*

Although the resistance was not yet fully characterized, it was known that the resistance transferred from *B. vulgaris* ssp. *vulgaris* (Holly) was based on the *Rz1* gene and from *B. vulgaris* ssp. *maritima* on the *Rz2* gene (Lewellen, 2004). These genes were probably in association with other minor factors (Biancardi *et al.,* 2002; Pferdmenges, 2007). The use of varieties which contained the *Rz1* or *Rz2* gene allowed content reduction in virus up to 6 X 10^4 times in comparison with the viral content in susceptible varieties (Acosta-Leal *et al*. 2008). Moreover, varieties with *Rz2* gene allowed better resistance than *Rz1* gene (Scholten and Lange, 2000). In the present study, the virus content was also lower in varieties bearing the *Rz2* resistance gene than in variety with *Rz1* or without resistance genes. Nevertheless, resistance-breaking isolates of BNYVV appeared in 2002 in France and USA (Tamada *et al.*, 2003; Rush and Acosta-Leal, 2007; Liu and Lewellen, 2007). It was possible that *Rz2* of *B. vulgaris* ssp. *maritima* provide only partial resistance to these isolates, which were significantly modified by so-called minor resistance genes of host plant (Kornienko *et al.*, 2011). In several greenhouse and field trials, it had been shown that certain minor genes in *Rz1* hybrids improved the resistance to rhizomania (Lennefors, 2006).
There is the actual risk of emergence and spread of new variants of BNYVV owing to reduced genetic diversity of cultivated beets and strong selective pressures exerted by the resistance genes *Rz1* and *Rz2*. The genetic diversity of varieties of sugar beet is the key for breeders to constitute new varieties in order to avoid genetic erosion with time (Palloix *et al.*, 2009). It is

consequently very important to avoid favoring the occurrence of RB strains of BNYVV (which reduce the pool of resistant varieties).

The aa valine had been associated in USA with the ability of BNYVV to overcome *Rz1* (Acostal-Leal *et al.*, 2010). Therefore, Acosta-Leal suggested that a specific p25-*Rz1* interaction may be required for disease development (deactivation or delay of *Rz1*-mediated defense responses) rather than defense activation like inhibition of PTGS by p14. Given the dominant inheritance of *Rz1*-mediated resistance, it was possible that the recessive *Rz1* allele lacks or encoded a defective defense mechanism, explaining why susceptible *Rz1* plants can be severely infected by haplotypes of BNYVV that encode alanine and several other amino acids. Others authors observed also resistance-breaking with the same tetrad or with others (Table 5.4).

Table 5.4. Tetrads associated with resistance-breaking.

Tetrads associated with RB	Associated BNYVV type	Authors
VCHG	A-type	Acosta-Leal *et al.,* 2007; Koenig *et al.* , 2009b
VLHG	A-type	Chiba *et al.* , 2008
AYHG	A-type	Chiba *et al.* , 2008 and 2011
AYPR	A-type	Bornemann and Varrelmann, 2013
AFHR	A-type	Chiba *et al.* , 2008 and 2011
AYHR	B-type	Chiba *et al.* , 2011
SYHG	P-type	Chiba *et al.* , 2011

BNYVV identity stability

It was well known that viruses rapidly mutated under environmental constraints. Though, the relative stability of BNYVV and especially of B- and P-pathotypes was well known, as well as the identity of nucleotide sequences to the samples described more than 15 years ago. But, on the other hand, a strong selection pressure was identified on the aa tetrad of the p25. For all the sequences around the world, by direct sequencing, there were 21 differences in the aa sequences between the p25 of A-, B- and P-pathotypes have been noted (21, 23, 28, 30, 32, 42, 49, 67, 68, 69, 70, 73, 118, 129, 132, 163, 179, 192, 198, 213 and 219). These different mutations were in relation with the type of tetrad and thus with the BNYVV pathotype.

One might speculate that such mutations would also have an impact on the p25 oligomerization and its intrinsic efficiency. Within the single BNYVV A-pathotype, 16 differences in the p25 aa sequences were recorded. Inside the B-pathotype, there were fewer differences with only aa 21 and 67. Inside the

P-pathotype, there were three differences with aa 21, 23 and 28. Koenig *et al.*, (2005) hypothesized that there were specific mutations of the virus accompanied by substitutions of aa in the coat protein CP, p25 and p26, leading to overcome host resistance genes. Even a single mutation was enough to create resistance-breaking strains (Koenig *et al.*, 2009b; Acosta-Leal *et al.*, 2010).

In this thesis study, it was shown that there was SNP within a same BNYVV pathotype between different varieties implemented in the same field. In general, for the p14, p25 and p26 of BNYVV there were between 94% and 100% of identity (matrix identity), at the aa level, in *Rz1* cultivar with weak resistance, *Rz1* cultivar with good resistance and *Rz1Rz2* cultivar. The genetic diversity for BNYVV was relatively limited as compared to other plant RNA viruses like *Potato virus Y* (Piche *et al.*, 2004, Acosta-Leal *et al.*, 2005). It was expected that the resistance-breaking isolates should emerge only from few mutations. In particular, it had been experimentally shown that overcoming of sugar beet resistance to rhizomania could be in USA the result of selection of mutant RNA 3 with a single mutation (aa substitutions at positions 67-70 and 198 in the protein p25) (Schirmer *et al.*, 2005, Koenig *et al*., 2009b. Acosta-Leal *et al.*, 2010).

BNYVV tetrad diversity

Beet necrotic yellow vein virus (BNYVV) was detected in 839 of the 1058 samples collected in France between 2009 and 2011 (79,3%). There was a high diversity in the Pithiviers area. B-pathotype BNYVV was detected most frequently. The p25 coding region on BNYVV RNA3 was amplified by RT-PCR and sequenced. Seventeen different variants of the highly variable aa tetrad at positions 67-70 of p25 were identified, i.e. AYHR (347 samples), SYHG (160 samples), AYHG (14 samples), AYPR (39 samples), TYHR (13 samples), AYHT (2 samples), AYHS (1 sample), ASHR (1 sample), AFHG (1 sample), SYHR (5 samples), VCHG (4 samples), SYHN (1 sample), AFHR (3 samples), VYHG (1 sample), SFHG (2 samples), AFLG (1 sample), VYHR (1 sample). The first two variants were found most commonly. It was tetrads from the B-pathotype and the P-pathotype, respectively. In 252 out of 737 BNYVV-positive samples, we detected P-pathotype BNYVV that had previously been identified only in France, Kazakhstan, Iran and recently in the UK. Some tetrads were also found in mixed infection (double or triple) in a single sugar root, i.e. SYHG-AYHR (83 samples), AYHR-AYHG (7 samples), AYPR-AYHR (17 samples), TYHR-AYHR (7 samples), AYHR-AYHT (2 samples), AYHR-AYHS (1 sample), AYHR-ASHR (1 sample), AYHR-AFHG (1 sample), VCHG-AYHR (2 samples), SYHG-SYHN (1 sample), SYHG-SYHR (4 samples), AYHR-AFHR (3 samples), SYHG-VYHG (1 sample), SYHG-SFHG (2 samples), AYHR-TYHR-AYPR (1 sample), AYPR-AYHR-AYHG (6 samples) and AYHR-TYHR-SYHG (1 samples). In Iran, Mohsen *et al.* (2009) detected also different tetrad variants (i.e. SYHG, ACHG, AHHG, AYHG, ALHG, AFHR, AFHG, AHYG, VLHG and VHHG) and saw higher tetrad diversity than have been reported from any other country. Only four tetrads are common between Iran and France (SYHG, AYHG,

AFHR and AFHG). It was three tetrads from the BNYVV A-pathotype and one from the P-pathotype. In Iran, none of the BNYVV B-pathotype or A-pathotype samples contained the fifth RNA species usually associated with P-pathotype BNYVV in other countries. In our study, however, we evidenced BNYVV B-pathotype and A-pathotype with the fifth RNA in a single sugar beet root, even in field where the P-pathotype was not detected.

Due to the high frequency of the tetrad AYHR and SYHG in the different samples, even sometimes in mixed tetrad infection in a single sugar root, the multiplex RT-mqPCR was developed. The results showed contrasted level of each tetrad in the different varieties analyzed. The tetrad SYHG showed the higher level.

P25 as Avr and virulent factor

The tetrads VLHG and VCHG were associated with resistance-breaking varieties in USA (Koenig *et al.*, 2009b; Acosta-Leal *et al.*, 2010). The tetrad VCHG was also detected in the variety bearing the resistance *Rz1rz1Rz2rz2* in France. Nevertheless, such tetrad was not associated with symptoms in the sugar beet fields. Borneman and Varrelman (2009) claimed that no tetrad was directly linked to the RB despite the high variability. The RB tetrad couldn't activate the mechanism of specific resistance. Chiba *et al.* (2008) and Schirmer *et al*. (2005) suggested that the aa 68 in p25 of BNYVV determines the resistance phenotype and the specificity of host genotype but that there were also other punctual mutations involved like in position 70 and 179. Chiba *et al.* (2008) proposed that the different response of various BNYVV isolates were due to the aa residues at position 68 in the p25 (Table 5.5). The protein p25 harboring phenylalanine-68 acts as a stronger elicitor than p25 proteins with tyrosine-68 or histidine-68 (Chiba *et al*., 2008). Tetrad with phenylalanine-68 had a stronger impact on the symptoms. Chiba *et al.* suggested the possibility that single amino acid changes (Phe, Tyr or His → Leu or Cys) at position 68, or amino acid changes at position 70 (Gly → Arg (R)) or 179 (Asn (N) → Asp (D)), may result in the occurrence of resistance-breaking virus strains. In this study in France, we found mainly the tetrad AYHR and SYHG. For these two tetrads, we had in aa. 68 the same tyrosine aa. The tetrad AFHG was found in only one sample, the tetrad AFLG in another sample and the tetrad AFHR in three samples. But, tetrads were not related to the higher virulence observed in the field. Such tetrads were commonly associated to the A-pathotype. However, only a limited of BNYVV A-pathotype was found in our study in comparison with much more BNYVV B-pathotype and P-pathotype. At the BNYVV p25 aa 70 position, there was an arginine (positively charged) in the B-pathotype and a glycine (non polar) in the P-pathotype. Such change implied a possible change of aa function. Similarly, at the position aa 179, there was also a change with an asparagine (polar non charged) in the P-pathotype and an aspartate (negatively charged) in the B-pathotype. Chiba observed that p25, acting as Avr factor, could be involved in the suppression of cell-to-cell movement rather than in virus replication. The p25 would possibly have a dual function as a virulence and Avr determinant.

Table 5.5. Amino acid of the tetrad (p25 protein) and their position.

Chiba *et al.* (2008)				In France			
aa67	**aa68**	**aa69**	**aa70**	**aa67**	**aa68**	**aa69**	**aa70**
A	F	H	G	A	Y	H	R
A	Y	R	V	S	Y	H	G
S	Y	H	G	A	Y	P	R
A	H	H	G	T	Y	H	R
A	L	H	G	V	C	H	G
A	C	H	G	A	F	H	R
A	Y	H	G	A	F	L	G
A	Y	H	R	A	F	H	G

Rem: The coding amino acids are the following: A-alanine, F-phenylalanine, H-histidine, G-glycine, Y-tyrosine, R-arginine, V-valine, S-serine, P-proline, T-threonine, L-leucine, C-cysteine.

Compartmentalization of BNYVV pathotype in sugar beet root

Doucet *et al.* (unpublished, 2006) showed a co-localization of BNYVV and *P. betae* in the cortex of sugar beet rootlets. They showed that even the A-, B- and P-pathotype infected rarely the xylem but well the phloem and the vascular parenchyma. The major BNYVV infected tissues in the rootlets observed were the vascular parenchyma and the phloem sieve elements, which favor the model translocation via the phloem. Moreover, they showed that there was a concentration gradient of the BNYVV P-pathotype. There were more cells infected by the P-pathotype in the endoderm and the phloem than in the epidermis and the cortex (in susceptible and partially resistant varieties). It was also the case for the A-pathotype but with a less importance. For the B-pathotype, the cells were homogeneously infected but the B-pathotype was more localized between the epidermis and the endoderm. This could explain the fact that the B-pathotype was more frequently found in the region of Pithiviers, when the analysis were made only with peripheral tissues. Moreover, the B-pathotype seemd in better relation with the vector than the other pathotypes. There were more cells infected by the P-pathotype than the A-pathotype and than the B-pathotype. This showed the better multiplication of the P-pathotype in the sugar root and was linked with the fact that, in this work, the ELISA value were often more important for the P-pathotype than the other BNYVV pathotypes.

Overall, for all the pathotypes, the epidermis was slightly infected. The virus did not move indifferently sideways. The results were different according to the resistance varieties used. There were three limiting barriers for the viral movement: the exoderm (localization of viral multiplication and translation), the endoderm, the regulation between the parenchyma cells and the companion cells adjacent to the sieve tubes. For the penetration, there was an infection of the adventives roots through meristem infection. There was an attractive zone for the vector just above the root cap which allowed acquisition and transmission (Doucet *et al.*, unpublished, 2006). The

evaluation of the viruses' abilities to spread over long distances within the plant and multiply within root tissues showed a ranking in aggressiveness among the pathotypes: B < A < P. The P-pathotype presented a higher ability to spread in the vascular cylinder. These results were fully in accordance with observations previously published by Heijbroek *et al.* (1999). They suggested that P-pathotype was more pathogenic and that it was also more rapidly transported through the vascular bundles. This pathotype was thought to be responsible of more severe rhizomania symptoms (Miyanishi *et al.*, 1999, Tamada *et al.*, 1996). The B-pathotype appeared to be less damaging than the A and P-pathotypes (Heijbroek *et al.*, 1999). A comparison of p21 and p75 RNA2 and p25 RNA3 ORFs sequences showed that the A-pathotype shared more identity with the P-pathotype and that within the A-pathotype variants existed (Meunier, 2005).

Doucet *et al.* (unpublished, 2006) showed already the heterogeneity in the infection of sugar root by several BNYVV pathotypes. In the present studies, we observed also result differences in the repetition of the analysis of the same sample. That was why all the samples were lyophilized (freeze dry), ground and homogenized before the analysis. This way the infectious profile of the entire sugar root was better analyzed. By direct *in situ* hybridization in the 3' end of all the five RNA's, we detected also, with a ssDNA wobble probe, the presence of BNYVV in different tissues in the root sections of several level of resistance varieties. The better period for sampling was around the harvest period because the root was more infected at the end of the culture, by more pathotypes and in higher concentration.
Through the study of root section of mature sugar beet, the BNYVV pathotype distribution in the taproot was observed. The results were different in the different section of a same root. Mixed infection of pathotype (tetrad: SYHG, AYHR, AYPR, TYHR, AYHG) were observed in *Rz1* cultivars with weak resistance and *Rz1* cultivar with good resistance, with a larger proportion of the B-pathotype. There was also mixed infection of pathotypes in leaves. BVQ was almost present in 100% of all the sections. BVQ seems to be homogeneously distributed in the taproot. There were heterogeneous distribution in the taproot for the fifth BNYVV RNA, the BNYVV RNA2 and BSBV. A complementary analysis was made on on one side the epidermis and on the other side the central root. In these different tissues, mixed infection of pathotype and the RNA3 was found both in the central and peripheral tissues. In the peripheral tissues and epidermis, there was slightly more infection of pathotype than in the central root for *Rz1* cultivar with good resistance. It was the opposite in the *Rz1* cultivars with weak resistance. Moreover, the BNYVV B-pathotype was more frequently detect in the peripheral tissues than in the central tissues. The P-pathotype was found more frequently in the central tissues. However, the fifth RNA was detected more often in the peripheral tissues than in the central root. The RNA5 was even never found in the central tissues. This RNA was more often detected in the *Rz1* cultivars with weak resistance than in the *Rz1* cultivar with good resistance. It was slightly the same for the RNA2.
Consequently, the best period to prospect the disease in the field was at the time of the harvest because the proportion of roots infected by BNYVV,

BSBV, BVQ and *P. betae* was more important. Another reason was because there was more mixed infection of BNYVV pathotype in a single sugar beet root which can provide recombination/reassortment in the viral genome. And finally, we could have a mixed infection of different viruses which provided interaction and complementation. In this sampling way, we had a better representation of the infection of rhizomania in sugar beet than in the beginning of the culture.

Differences between France and USA

In USA, a single U/C nucleotide substitution changing alanine to valine in the BNYVV p25 protein promoted increased virus accumulation in roots of mechanically inoculated, partially resistant sugar beet seedlings (Koenig *et al.*, 2009b; Acosta-Leal *et al.*, 2010). The changes in the intra-isolate genetic structure of *Beet necrotic yellow vein virus* populations were associated with plant resistance breakdown (Acostal-Leal *et al.*; 2008). In the present study, no such single nucleotide substitution had been evidenced in the assays implemented with several resistance varieties. The origin of resistance-breaking in France might come from the occurrence of several parameters rather than a unique one. No recombination was evidenced between BNYVV pathotypes. But, the importance of the B-pathotype in the assays followed by the increase of the P-pathotype in the region of Pithiviers was evidenced. There were also very high infectious potential in the majority of the assays studied and an important presence of BVQ and BSBV associated with BNYVV in a single sugar beet root. No BBSV and BSBMV were identified in the assays. The intense culture of sugar beet in the region of Pithiviers might cause high pressure on the virus during many years and might have favored the fitness of BNYVV P-pathotype with the fifth ancestral RNA (Chiba *et al.*, 2011). Tamada *et al.* (1996) showed evidence that *Beet necrotic yellow vein virus* RNA5 is involved in symptom development of sugar beet roots.
In the samples collected, the BNYVV was highly present with 79,3% of sample infected. BVQ was found in 66,7% and BSBV in 61,7%. There is consequently a large proportion of these three viruses in the samples from Pithiviers. The incidence of BSBV and BVQ was found at a lower level in other countries like Iran, Belgium and USA. In France, the fifth RNA was present for 41,2% of the samples analyzed.
In USA, Wisler *et al.* (2003) showed the interaction between BNYVV and BSBMV. The Rz resistance gene to BNYVV did not confer resistance to BSBMV. Moreover, contents of BSBMV were significantly higher in single infections than in mixed infections with BNYVV, in both the rhizomania-resistant and susceptible varieties. In contrast, contents of BNYVV were high in single and in mixed infections in the rhizomania-susceptible variety, but were low in the rhizomania-resistant variety. BNYVV may either out-compete or suppress BSBMV in mixed infections, even in rhizomania-resistant varieties in which contents of BNYVV are extremely low. Weiland *et al.* (2007) reported about the occurrence of BBSV in the USA that displayed severe symptoms and influenced sugar beet growth similar to rhizomania. However, BSBMV and BBSV were not found in the samples analyzed in France. But, the interactions between these viruses triggered the question of

the possible same comportment between BNYVV, BSBV and BVQ present in France.

BNYVV and dispersion of P-pathotype to the East

The BNYVV P-pathotype was the second more present in the assays. But, in the region prospect, it was the more present. This pathotype had a dispersion to the East of the Pithiviers region (Figure 5.2). The P-pathotype with the tetrad SYHG and the fifth RNA had been linked to high level of ELISA values in the samples from the assays implemented. The fifth RNA was present for 41,2% (of 1058 samples analyzed) and was probably localized in the peripheral tissues in the root. This ancestral pathotype could have emerged few years ago in the new cultivars and have exacerbated the symptoms with its fifth RNA in synergism with p25. Tamada hypothesized a virus with 5 RNAs, loosing it to go for 4 RNAs. He also mentioned that probably the virus was not originally on sugar beet. Such data supported such hypothesis, as well as publications related to P-pathotype without RNA5. Since, the resistance-breaking in 2002 in France in *Rz1* cultivars, it was tempting to speculate that different isolates of the BNYVV P-pathotype could have improved fitness allowing them to spread over in the region. Nevertheless, it was also useful to stress the lengthy process of BNYVV, with a short distance spread based on zoospore propagation with the help of the free water present in soils (the area of Pithiviers, with irrigation and soil conditions favorable to the infection) and long distance spread through machines or with the help of man. Looking at the spreading shape of the map generated with P-pathotypes, it was also tempting to link it with the location of the different sugar refineries around Pithiviers, where soil collected with the beets were gathered and concentrated.

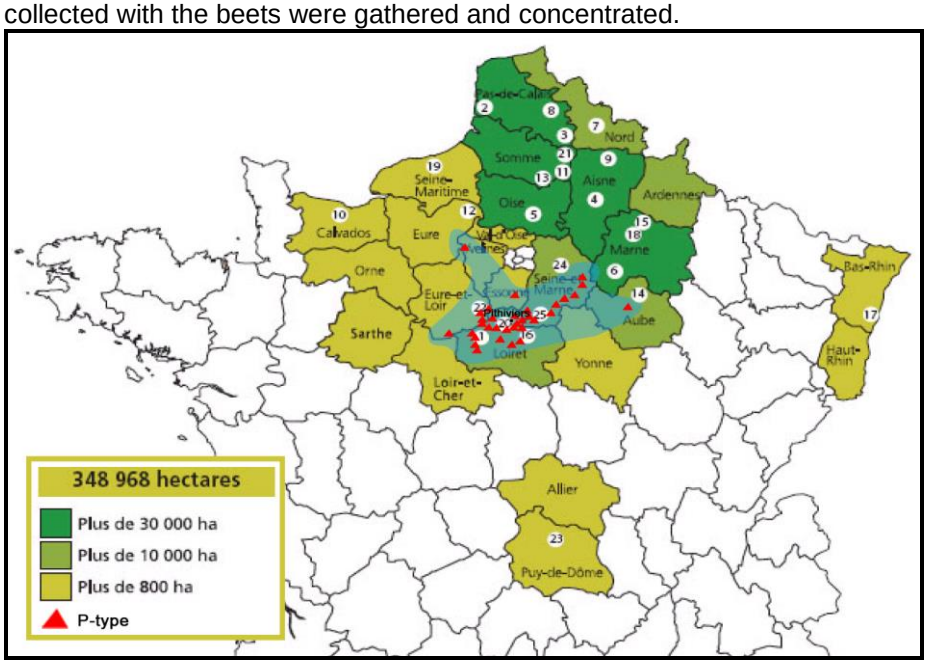


Fig. 5.2. Dispersion of the BNYVV P-pathotype around the Pithiviers area. The culture of sugar beet is mainly situated in northern France. In 2008-2009, 29 departments have cultivated sugar beet and it represents 348 968 hectares (2,1% of the utilized agricultural area). The production was 29 million tones of sugar beet with 18,9% of sugar content. The two most important departments are “Aisne” and ”la Marne”. The coded sugar refineries in the studied area are the following: 1-Artenay, 6-Connantre, 12-Etrepagny, 14-Arcis-sur-Aube, 16-Corbeilles, 20-Pithiviers, 22-Toury, 24-Nangis, 25-Sources-sur-Loing.

Pomoviruses associated

The rhizomania syndrome of sugar beet and its causal agent, BNYVV, had been widely studied since the 1970s (McGrann *et al.*, 2009). However, the involvement of BVQ and BSBV in the disease, often associated with beet infected with BNYVV, had never been clearly established and no particular interest in understanding their specific functionality was followed (Crutzen *et al.*, 2009a). In the present study, the level of BNYVV (RNA3) was the highest with 79,3% (of 1058 samples) in average (2009-2011), followed by BVQ with 66,7% and BSBV with 61,7% (of 847 samples analyzed). These high proportions of BNYVV and *Pomoviruses* associated detected in the samples can be linked to the high level of *Polymyxa betae* in the French soils (high level of MPN). The proportion of roots infected by BNYVV was more important at the time of harvest compared to the beginning of culture. Moreover, it appeared that the number of mixed infection was also higher in the harvest period which can provide recombination/reassortment in the genome. Moreover, it could have interaction and complementation between BSBV, BVQ and BNYVV due to the virus mixed infection in a single sugar root.

BSBV and resistance of *Rz1rz1Rz2rz2* varieties

The influence of the double resistance varieties were observed against the presence of BSBV in the sugar beet. When the *Rz1* cultivars with weak resistance and good resistance were highly infected by BSBV in a field, the *Rz1Rz2* cultivar was no or less infected by BSBV. In general, the double resistance variety was infected in the beginning of the culture by BSBV but was almost not infected by BSBV at the harvest period. Globally, for all the *Rz1Rz2* cultivars analyzed in May and September, there were only 27% of the *Rz1Rz2* cultivars samples infected by BSBV while there is 61, 7% of the BSBV present in the varieties from all the samples investigated. There was consequently an influence of the *Rz2* gene on the presence or not of BSBV in sugar beet.

High level of virus and vector MPN

Some soils in Europe might show high inoculum density of viruliferous *Polymyxa betae*. Moreover, the B-pathotype seemed preferentially transported by the vector. The content of P-pathotype in the tap root of sugar beet was also more important than A- or B-pathotype infections. This indicated that the P-pathotype moved more rapidly in the plants than the two other BNYVV pathotypes which were more localized in peripheral tissues

where the vector was fixed for cell infection (Heijbroek *et al.*, 1999). Pferdmenges & Varrelmann (2009) explained that the independent geographically RB selection followed only the uniform culture of *Rz1* sugar beet and was independent of the infectious potential. In the present study, the soils selected were highly homogeneous for the tetrads found in the field but there were some field highly heterogeneous which presented the three pathotypes in the same field. There were also few fields without any BNYVV and consequently there were no infectious potential. The detection of the tetrad diversity through the study of the infectious potentials was less efficient than the study of root samples from the field. But, the detection of the presence of BNYVV with *Rz1* susceptible variety in the study of infectious potential was more efficient than the study of root samples from several kinds of varieties in the field. Goffart *et al.* considered high infection potential when the IU/gr of soil was higher than 13,6 (20 repetitions per dilution). The higher vector MPN observed in 1990 in Belgium was 123,3 IU/gr of soil. On 52 different fields studied in Belgium by Goffart *et al.*, the majority of all the sugar plants infected by *Polymyxa* were no more infected in the dilution 1/3125 while they were again after the dilution 1/78125 in France between 2009 and 2011. Besides, on 19 different fields studied in France, the MPN range for virus was between <0,023 and 680 IU/gr of soil. And for the vector, the MPN was between 47 and >23000 IU/gr of soil (12 repetitions per dilution). In comparison with the results obtained by Goffart, we can conclude that the French fields are under very high infection potential. There was also an increase of the inoculum potential during a beet season due to the presence of the culture of sugar beets in the field. Overall, for the ten field investigated, the virus MPN and the vector MPN were weaker in May 2010 compared to September 2010. The culture of sugar beet in short rotation increased consequently the risk of a probable resistance-breaking event. However, we observed a decrease of the virus MPN in field planted with *Rz1rz1* varieties that were also resistant to nematode. The resistance gene against the nematode could have also an impact on the viruliferous *P. betae* in the soil. If *Rz* and *N* (nematode) resistance are on the same chromosome in beet cultivar, *N* resistance in could be in interaction with the nearby *Rz1* resistance. Moreover, in case of mixed BNYVV pathotype infections, these varieties showed lower root severity than the other varieties (*Rz1rz1* without nematode resistance and *Rz1rz1Rz2rz2* varieties). This result was surprising beacause the *Rz1 + N* partial resistant varieties were the first showing resistance-breaking events in the field. The hypothesis was that the nematode resistance gene decreased the resistance to rhizomania. However, our results showed a decrease of the virus inoculum in the soil with *Rz1 + N* varieties. These varieties may have maybe a greater selection pressure on the virus and it could explain the early resistance-breaking events.

Inter genera viruses' competition

It was known that the temperature influenced the virus replication rate in the plant. BSBV was the virus that was developing the faster. Consequently, in the sugar beet season, the BSBV infected the plant firstly in spring and the

infection of BNYVV increased in July when the temperature was higher. Prillwitz and Schlösser (1992) showed that preinfection of sugar beets with BSBV-2 reduced significantly the BNYVV content but increased significantly the number of cystosori. It could have firstly a competition between different viruses early in the season. Sometimes, BSBV and BNYVV are in competition. When one increased, the other one decreased (Prillwitz and Schlösser, 1993). Either BSBV or BNYVV infecting in first time avoided the overinfection of the second in the same sugar root. There was consequently an effect of crossprotection. This was the case for BSBMV and BNYVV through a repression of structural proteins through one of the two viruses (Mahmood and Rush, 1999; Workneh *et al.*, 2003). In the present study, we found the two viruses in mixed infection in several samples but the presence of BNYVV (79,3%) in the samples was slightly more frequent than BSBV (61,7%). BSBV was less detected in varieties with the double resistance gene (*Rz1rz1Rz2rz2*), which showed also lower root severity.

Intra species virus' competition (reassortment or satellite?)

Moreover, competition between the BNYVV B-pathotype associated with the tetrad AYHR (p25) and the P-pathotype associated with the tetrad SYHG (p25) was evidenced, at least for BNYVV RNA3. Firstly, we demonstrated that the tetrad SYHG was linked to higher CP accumulation as revealed by higher ELISA values compared to the tetrad AYHR. The tetrad SYHG allowed a better virus accumulation and multiplication inside the sugar beet root. There was a modulation in the production of each RNA. There was a determination coefficient for 70-80% between the tetrad-type coded by RNA3 and CP coded by RNA2. When AYHR content increased strongly, the CP content increased slightly. But, when SYHG content increased strongly, the CP content increased also strongly. When there was SYHG and AYHR mixed together, the CP content remained constant and low. The mixed tetrad infection influenced the level of SYHG in the sugar beet root and its multiplication. There was a cross-protection between the different BNYVV pathotypes. Our explanation was that there was a double balance between virus-vector and virus-plant in the selection of the tetrad/virus type. The tetrad had an impact on the disease. In term of transmission and conservation in the soil, the tetrad AYHR/B-pathotype was known to be better linked with its vector than the tetrad SYHG. The ELISA and IP results confirmed it. The tetrad SYHG/P-pathotype movement and multiplication was favored in the plant. Some vector contained sometimes the tetrad SYHG and when the vector infected the roots, the tetrad SYHG compensated for its poor relationship with its vector showing a better ability to multiply itself in the taproot. However, it was not the case for the tetrad AYHR. Consequently, if such hypothesis was verified, one could expect that an increase in the rotation period would imply a decrease in the level of the tetrad SYHG presence in the field and the sugar roots. Simply because the MPN and the accumulation in the vector of the tetrad SYHG was much lower than what was observed for the tetrad AYHR. In conclusion, the level of rhizomania was certainly influenced by the competition between the different

BNYVV pathotypes, and possibly by competition between BNYVV and pomoviruses.

In 20% of the samples analyzed through a single sugar beet root, tetrads associated to the B-pathotype or the A-pathotype with a fifth RNA have been evidenced. Due to the high frequency occurrence, we suspected reassortant strains with the fifth RNA rather than RNA5 to behave like satellite (Table 5.6), as suggested initially by Bouzoubaa et al., 1985 and Kiguchi *et al.* (1996). Koenig *et al.* (2008) suggested BNYVV genome reassortment in several resistant sugar beet varieties with strong rhizomania symptoms. The fifth RNA was detected either with A-pathotype RNAs 1 to 4 or with a mixture of B-pathotype and P-pathotype RNAs. Peltier *et al.* (2008) had found the RNA5 with European BNYVV A-pathotype or B-tpathoype and also with the P-pathotype associated SYHG. BNYVV RNA5 was defined by several authors as a BNYVV satellite-like RNA5 in the category of large single stranded RNA satellites (Bouzoubaa *et al.*, 1985, Tamada *et al.*, 1989, Kiguchi *et al.*, 1996) (Table 5.6).

Table 5.6. Comparison between reassortant strain and RNA satellite.

Reassortant strain	RNA satellite
1) Viruses containing two or more pieces of nuleic acid (segmented genome)	1) Subviral agent: smaller than viruses but with some of their properties
2) Produced in cells coinfected with different strains of a given virus	2) Depend on co-infection of a host cell with helper virus for productive multiplication
3) The segments act like mini-chromosomes, and each time a virus is assembled, it requires one copy of each segment.	3) Helper virus: virus used when producing copies of a helper dependent viral vector which does not have the ability to replicate on its own
4) The new reassortant strain will share properties of both of its parental lineages	4) Their nuleic acid have substantially distinct nucleotide sequences from either helper virus or host and there is little sequence homology
5) Reassortment is responsible for some of the major genetic shift in the history (influenza virus)	5) Large stranded RNA satellite: satellite with genomes that are 0,8 to 1,5 kb in size
	6) Encodes a non-structural protein that, at least in some cases, is essential for satellite RNA multiplication
	7) Some satellites can be exchanged among different helper viruses
	8) These satellites rarely modify the disease induced in host plants by the helper virus

Ref: National Center for Biomedical Ontology; BioNyt; Gerlach *et al*., 1986; Ni and Kemp, 1992; Kigachi *et al.*, 1996; Alberts *et al*., 1997; Mayo *et al.*, 1999; Fauquet *et al.*, 2005; Hood, 2006; Krupovic and Cvirkaite-Krupovic, 2011.

Micro-evolution and selection pressure

Selection pressure varied greatly depending on the BNYVV gene and geographical location. Isolates of Italy strain, in which p25 was subjected to the strongest positive selection, were able to overcome the *Rz1*-host

resistance gene to differing degrees, whereas other geographically limited strains could not. Resistance-breaking variants were generated by p25 aa changes at positions 67 and 68 (Chiba *et al.*, 2011). The aa 68 in p25 was demonstrated to control certain virus-specific resistance interactions (Chiba *et al.*, 2008). Moreover, in USA, Acosta-Leal and associates (2008) reported positive selection of aa 67 and 68 in RB isolates. Isolates with valine at position 67 induced more severe symptoms in resistant cultivars than the wild-type isolate with alanine (in the same BNYVV pathotype) at that position (Acosta-Leal *et al.*, 2008; Koenig *et al.*, 2009; Pferdmenges *et al.*, 2009). If we compare this with what happen in France, we were tented to think that the tetrad SYHG with the fifth RNA linked to the P-pathotype was responsible of the resistance-breaking in France compared to the tetrad AYHR from the B-pathotype. Bornemann and Varrelmann (2013) showed by deep sequencing that the plant genotype exerted a clear influence on the frequency of RB tetrads. In *Rz1* plants, the RB variants outcompeted the WT variants, and mostly vice-versa in susceptible plants, demonstrating a relative fitness penalty of RB mutations. The strong genotype effect supported the hypothesized *Rz1* RB isolate selection with four RNAs, suggesting that a certain tetrad needed to become dominant in a population to influence its properties. Tetrad selection was not observed when a RB strain, with an additional p26 protein encoded by a fifth RNA, competed with a WT strain, supporting its role as a second BNYVV pathogenicity factor and suggesting the reassortment of both pathotypes. In our study, we found pathotype reassortment in sample with mixed pathotype infection where the more present (distribution in fields) tetrad AYHR was in mixed infection with SYHG which was the more multiplied in the roots (higher content).

Chiba *et al.* (2011) showed that the mean nucleotide diversity of all BNYVV isolates for the four sequenced genes was relatively low (0,016 to 0,025) and that of the within-group of BNYVV isolates was even lower (0,002 to 0,018), suggesting that BNYVV populations were genetically very stable or have diverged recently. Chiba and associates showed also that the values of d_{NS}/d_S (pairwise synonymous substitutions per synonymous site / nonsynonymous substitutions per nonsynonymous site) in Italy differed greatly with BNYVV genes p25 (1,500), p31 (0,500) and CP (0,333). The p25 gene was subject to very strong positive selection. The values for each gene of the Italy strain were higher than those for other groups. Chiba suggested that the existence of the highest selective pressure on p25 may influence selection pressure on the other genes. Alternatively strong selection pressure may be imposed in parallel on all BNYVV proteins. And such strong positive selection pressure on the p25 gene was associated with cultivation of resistant cultivars.
In this study, only the micro-evolution in the P-pathotype was investigated. The analysis of the selective pressure of BNYVV was performed using between 129 and 495 sequences for p14, between 445 and 500 sequences for p25 and, between 382 and 500 sequences for p26. We showed that the values of positive selective pressure differed also greatly with BNYVV genes and with the variety investigated. The p14 gene was most constrained (more aa under high positive selection pressure) with ω values varying from 0,680

to 9,738 according to the aa and the variety. The p25 showed more aa under positive selection pressure but with less high ω values. The analysis revealed the occurrence of strong positive selection on some of the codons. The p26 showed also several aa under high selection pressure with the highest value (ω = 11,625) in the *Rz1* cultivar with good resistance. In the position 81 of p14 (PTGS inhibition), the ω values range observed for the different varieties was comprised between 6,294 and 9,738, which is under very strong positive selection pressure. This mutation was in a lysine-rich polar and positive charged region. It is a basic region which could have interaction with DNA (negative charged). The pattern of this sequence is $_{70}$**K**CCR**K**L**K**C**KK**QN**K**N**H**S**KH**$_{87}$. All these non-synonymous mutations can directly affect protein conformation and function.

Deep-sequencing showed that there were different variants for the same pathotype of BNYVV (P-pathotype) from the same field according to the different kind of variety implemented (*Rz1* cultivar with weak resistance, *Rz1* cultivar with good resistance and *Rz1Rz2* cultivar). There were, for the three genes analyzed (p14, p25 and p26), functional changes in the aa mutation. The p25 presented more aa in positive selection pressure than the p14 and p26. For the three genes, it was the variety R1*Rz1Rz2rz2* which presented less aa. in positive selection pressure compared to the variety *Rz1* cultivar with weak resistance and good resistance. But, it was the p14 which had more high pressure levels compared to the two others genes analyzed. According to the variety genotype, the aa in selection pressure was not the same except for certain aa. like aa 81 and 88 in the p14; aa 27, 92, 94, 101, 108, 109, 160 and 176 in the p25; aa 23, 47, 61, 105, 153, 154 and 170 in the p26. The BNYVV P-pathotype consequently micro-evolved within-host during a single sugar beet crop season and changes a little according to the different varieties implemented in the field.

Conclusions

The contributions of this thesis in the understanding of the rhizomania complex in the Pithiviers area were numerous. Firstly, we brang an original methodological approach to study BNYVV and others viruses in the sugar beet and in the field. We evidenced intra-beet diversity and inter beet diversity for the virus and the vector. Intra-field diversity and inter field diversity in the Pithiviers area was revealed. Consequently, the spatial and temporal distribution of the BNYVV at the field level was better known. We had also evaluated single sugar beet taproot and evidenced diversity according to several sections in the root. Moreover, the effect of the sugar beet season on the virus and vector accumulation in the plant and in the soil were better characterized. The season allowed an increase of the virus inoculum in the soil and a higher increase of the vector inoculum in the soil. The sugar beet season brought also a higher diversity in the sugar beet at the end of the season. There was a very high diversity of BNYVV in the Pithiviers area and the tetrad SYHG related to the P-pathotype was found also outside the Loiret department. According to the varieties implemented in a homogeneous field infected by the P-pathotype, we evidenced micro-evolution of the virus in a single sugar beet taproot due to the selection

pressure. This selection pressure was observed on the p14, the p25 and the p26 in specific positions. Mixed pathotype infections were also evidenced suggesting reassortment events or satellite events as a possible cause of the resistance-breaking events. We evidenced competition between the tetrad AYHR and SYHG in case of mixed infection. The level of these tetrads was lower in case of mixed infection. Mixed infection of the rhizomania associated viruses in a single sugar beet was also evidenced. The varieties with *Rz1rz1Rz2rz2* resistance genes showed an effect on the control of BSBV. Theses varieties showed also lower symptoms of the disease compared to the others. The varieties with nematode resistance were also able to control the virus inoculum of the soil but not the vector inoculum.

The Pithiviers area was a rare region where we can encountered the three BNYVV pathotypes. We found more diversity in this region compared to somewhere else. And a lot of seed companies from all around the world had tested their different varieties in this specific region. There was consequently a high diversity of *Beta vulgaris* cropped in the Pithiviers area. Moreover, the increase of the culture of sugar beet for the sugar production in France began in 1806 with the Emperor Napoleon. The France had consequently a long history of sugar beet production. The introduction of BNYVV in the field might be maybe due to the cultivation of mulberry trees for silkworm culture. The irrigation was also frequently used in French field. All these parameters gathered together could explain the disease problems in this region. The results from the thesis suggested that the extension of the rotation was still a good practice to avoid disease problems. We advocated to the breeders to carefully use frequently the variety with *Rz1* alone due to the weak durability in the use of a single resistance gene. The frequent use of the *Rz1* variety can induce a high pressure selection on the virus and create resistant-breaking events. Varieties with several resistance genes (*Rz1* & *Rz2*) and with minor genes allowed higher effectiveness against the virus and the vector. Theses varieties avoided firstly the genetic erosion of the variety and the possible emergence of resistance-breaking events. These varieties must be used in field with high virulence and high virus content to decrease the level in the field. But, we shouldn't implement *Rz1Rz2* varieties in all areas to preserve the durability of the *Rz1* varieties. If we multiply the genetic diversity of the plant, we take the risk of seeing more quickly a mutant by-passing the *Rz1* resistance. We must also be careful because the double resistance could have a higher selection pressure on the virus. And in short term, this kind of variety could have a shorter durability. The best practice in high rhizomania area is to alternate the *Rz1* with the *Rz1Rz2* varieties to preserve the durability of the resistance genes. And we should better know the role of each resistance gene in the resistance.
The high diversity in the Pithiviers area compared to other countries could be also explained by other factors as the type of methodology, the technique of detection, the field level,… Indeed, in Kazakhstan, there was also high diversity for the BNYVV tetrads. In USA, Acosta-Leal did not use the same methodology than us to study the diversity of BNYVV. If we used this methodology in USA, perhaps a greater diversity in the BNYVV may appear. Even questions were still open, an explanatory model was proposed.

Basically, there were two drivers for the BNYVV diversity: the host plant, the sugar beet and the vector, *P. betae,* each playing the role of a filter, a bottleneck in the BNYVV evolution. The varieties implemented in the field generated variants in the soil and the vector filtered these variants and played a key role, together with man, in the spatial dispersion of the virus. The figure 5.3 presents such model for the emergence of resistant-breaking strains according to the varieties implemented and the different pathotypes present in the soil.

The figure 5.3 is explained here: if there is only the P-pathotype in the soil, the level of BNYVV in the varieties will be very high in the susceptible variety. When a *Rz1* variety is implemented, the level decreases. If another *Rz1Rz2* variety is coming, the level decreases even more. These varieties induce a selection pressure on the virus. The resistance of *Rz1* varieties has more chance to be overcome quickly by the virus than the resistance of *Rz1Rz2* varieties. If the virus finds the key of the *Rz1* resistance gene (with only one mutation for instance), the level of the new RB strain increases again and the RB strain shows strong symptoms on the plant.
If there is only the B-pathotype in the soil, the level of BNYVV in the varieties will be lower in the susceptible variety compared to the level with the P-pathotype. This BNYVV pathotype is the most common in the region of Pithiviers. The B-pathotype has the ability to multiply in the plant without showing strong symptoms on the plant and the plant has the possibility to control the B-pathotype. If a *Rz1* variety is implemented, the level of the virus will decrease. A similar effect is expected for *Rz1Rz2* varieties and *Rz1* varieties with nematode resistance. But, due to the selection pressure of one dominant gene (*Rz1*), new RB strains can emerge (with a single mutation, for example). The level of the B-pathotype then increases again showing more symptoms to a new equilibrium between the virus and the plant.
If there is mixed pathotype infection in the soil, the model becomes more complex. If mixed pathotype infection with only the A- and the B-pathotype are present, then the level of infection is lower than in case of mixed infection with the A-, B- and P-pathotype in susceptible plant. When a *Rz1* variety is implemented, the level decreases and induces a selection pressure on the three pathotypes in mixed infection. The diversity of the virus increases. More tetrads are observed. With a *Rz1Rz2* variety or a *Rz1* variety with nematode resistance, the level of BNYVV decreases even more. And, the diversity of the tetrads increases also more due to the selection pressure of two dominant genes. The virus tries more to find the key. When there is only the virus in mixed infection with the A-pathotype and the B-pathotype, the level is even lower again. Finally, the new RB strains could be a reassortment of the different pathotypes of BNYVV. And the level of these new RB strains increases again when the virus has found the key.
The model proposed here was based on the different results obtained when there were statistically significance differences between the parameters. In the majority of the results, this model was supported by the data. However, some particular cases don't follow this model. Others parameters not evidenced in the work were probably in cause for this.

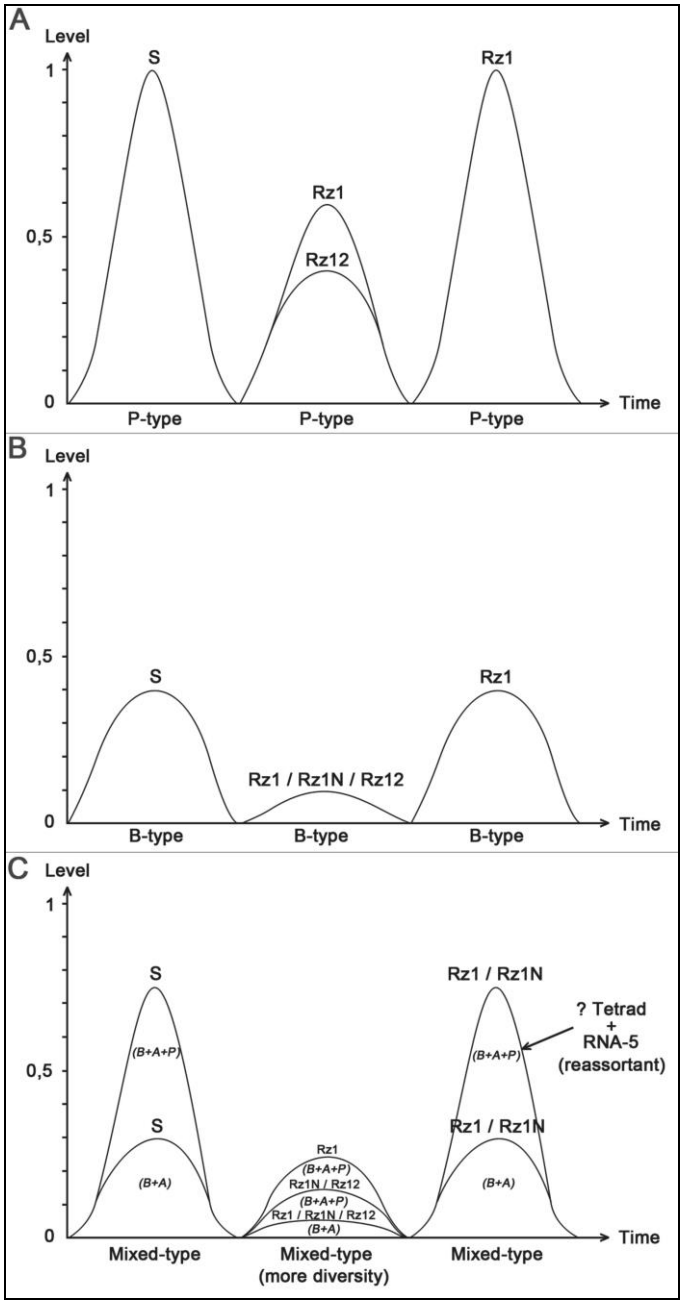


Fig. 5.3. Model for the occurrence of resistance-breaking strains according to the pathotype present in the field and the variety implemented in the field. The coded varieties are the following: S for susceptible, *Rz1* for *Rz1* resistance gene, *Rz1*N for *Rz1* and nematode resistance genes, *Rz1*2 for *Rz1* and *Rz2* resistance genes.2. A. ELISA level of BNYVV P-pathotype infection as a function of the successive varieties implemented in the time in a field only infected by the P-pathotype. 2. B. ELISA level of BNYVV B-pathotype infection as a function of the successive varieties implemented in the time in a field only infected by the B-pathotype. 2. C. ELISA level of BNYVV mixed pathotype infection as a function of the successive varieties implemented in the time in a field infected by the mixed pathotype.

Perspectives

In 2012, in Berlin, the genome sequence of sugar beet (18 chromosomes) was identified with support from BMBF (Federal Ministry of Education and Research in Germany). The genome of sugar beet was very different from the others plants already sequenced. This discovery will allow new knowledge for the structure and the function of this plant. If we know better the resistance genes, we will better understand the interaction between the resistance genes and the virus genome. Sugar beet decoding will now allow an accelerated breeding more efficient, accurate, and focused. It will also allow getting varieties of sugar beet stronger and more productive. It will be able to target specific regions of the genome for annotation in order to find potential candidate genes. It also allows identifying sequences and regulatory genes not yet described in the sugar beet to date. For important mechanisms, this may lead to the identification and rapid isolation of candidate genes that are studied by overexpression or with the help of knock-out approaches using transgenic techniques. The selection of a single major gene must be outcaste and breeders should juggle with different kind of resistance (minor resistance genes, several major resistance genes against viruses, resistance genes against vector or other pathogens, PTGS...) trying to keep the production potential of the sugar beet and the durability of these panels of resistance. Based on some differences in response to pathotypes, an important element in breeding resistant varieties will be to take also into account the pathotype of BNYVV used in specific selection programs. Disease management must also be improved to avoid dissemination of rhizomania complex. Breeders must be careful before implement transgenic resistance varieties and varieties from a restrictive selection.

The study of the infectious potential of the soil can be also improved. Recently, Isabelle Renaudin (2011) presented a poster about the evaluation of several direct extraction technique of the BNYVV from the soil. The successful extraction method retained (specific, sensible and accurate) was the Powersoil Total RNA isolation Kit Mobio (Ozyme). For the next analyses, it would be interesting to test this kit for the different soils investigated. It could save a lot of manipulation and preparation time for the detection of the virus direct from the soil. Consequently, direct multiplex quantification of the virus and the vector in the soil could be done.
If bioassays must be still used, then it would be interesting to increase the number of repetition per zone to obtain MPN values per zone more conclusive. We observed also a threshold in the detection of *Polymyxa betae* in the last dilution (1/78125). Consequently, it would be interesting to make a eighth (1/390625) or even ninth (1/1953125) dilution to see if we observe again the vector until these dilutions. Unfortunately, this represents more samples to analyze, more time, more means and more money.

Deeper work could also be made on the associated virus to study their interaction with BNYVV and their real impact on the varieties implemented in the field. The double resistance gene (*Rz1rz1Rz2rz2*) seems have a better

control of BSBV than BNYVV or these varieties could simply be a better control of BNYVV by *Rz1rz1Rz2rz2* leading to less root severity. And, the root severity with this variety was lower. But, we would have assumed that it would have been more severity if the preinfection of BSBV decreased and allowed later a better infection by BNYVV (due to the lack of cross-protection). But, it was not the case. It seemed that the associated viruses of rhizomania played an important role in the disease in France.

With regard to the ELISA, next analyses should be done with a correct negative control which is the uninfected plant. The ELISA value of the infected plant is consequently the optical density of the infected plant minus two times the optical density of the uninfected plant (the blank) (Nahm and Goldblatt, 2004). In this way, the absorbance of the plant is substracted and there is only the absorbance of the disease. All the repetitions for one sample should be done in the same time to avoid bias. And the blank should be analyzed for each plate.

It would be also interesting to obtain the data of the yield in each varietal band. We have still no idea of the link between the ELISA values or the root severity values or other parameters tested with the yield. Besides, it would be also interesting to obtain the data of the root severity in each zone of the field to make a direct link without bias between the results and the symptoms. And the use of a susceptible variety (Encarta, Cadyx) in the same zone of the other varieties tested would be useful to compare the results in the behavior of each variety in the same place. Indeed, in this work, it was very difficult to link the results with the symptoms observed in the field.

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APPENDICES

APPENDIX A

Complement information for materials and methods

A.1 Preamble

In order to detect the BNYVV RNA3 and RNA5 and the vector *Polymyxa betae* from field collected plants and soil samples in France, methodological questions regarding the efficiency, the repeatability and the sensitivity of the analyses were addressed for optimization. The sampling was made randomly in each zone. A visual evaluation for the symptoms incidence was given for each field. Moreover, the root severity was measured for each zone and each varietal band. And different parameters have been also measured in each zones (nematodes cyst and larva, composition of the soil, pH, temperature and precipitation). We suggest here to homogenize the samples to detect the virus in the integrality of the sugar beet roots. The real sensitivity and efficiency of the PCR was investigated. The incidence of viruliferous BNYVV through the infectious potential in France was evaluated using a bio-assay with susceptible (*rz2rz2rz1rz1*) beet cultivars with limit dilution of soil from each field. Sofia and Cadyx were grown in these soils coming from the region around Pithiviers. The effect of a beet culture in the field rotation between sawing and harvesting was evaluated through the diversity of the virus and the presence of the vector but also for the variation in the infectious potential. The virus/vectorial heterogeneity and distribution in the beet and in the field were analyzed to improve the sampling method and analysis. The real-time quantitative RT-mqPCR (RT-PCR multiplex quantitative) was developed as a tool for evaluating the content of BNYVV pathotype in a cultivar and for evaluating the resistance to two specific pathotypes by quantifying the virus concentration. The global content of the virus in each sample was also evaluated through ELISA.

A.2 Introduction

The first detection of the virus BNYVV began with the ELISA technique in the 90's which is focused on the viral capside detection (Kuszala *et al.*,

1986; Heijbroek *et al.*, 1988; Tamada *et al.*, 1989). Tuitert *et al.* (1990) and Goffart & Maraite (1991) quantified BNYVV and *P. betae* by assessing the infectious potential in soil using the most probable number method (MPN) (Halvorson and Ziegler, 1993; Cochran 1950; Kleczkowski, 1968). Tuitert used a bioassay with serial dilutions of the soil for the quantification. The numbers of positives per dilution were related to the number of infective units of the organism in the original sample. After the 90's, new technologies focused on the viral RNA allowing to enhance the precision of the analyses. Four different viral pathotypes have been described initially by RFLP, then based on major sequence divergence (Kruse and Koenig, 1994; Schirmer *et al.*, 2005). The A-pathotype was found in all European countries, in Iran, in USA, in China and in Japan and the B-pathotype was particularly detected in France and in Germany, but also in Switzerland, in Suede, in China and in Japan. The J-pathotype was localized in Japan and China while the P-pathotype was found in France, Kazakhstan (Hleibieh *et al.*, 2007; Stacey *et al.*, 2003; Koenig and Lennefors, 2000) and recently in UK (Lemaire *et al.*, 2003). Meunier *et al.* (2005) was the first to use the RT-PCR technology for the detection of BNYVV. This last technology allowed a better precision and efficiency.

In the beginning, the work of the thesis consists in studying the methodological approach for virus and vector dosing. We found the best and most representative procedure and found the parameters to take into account. Systematic and repetitive results were established for individual sugar beet root analysis. As Heijbroek *et al.* described already in 1999, some partially resistant cultivars varied in their response to the occurrence of different pathotypes of *Beet necrotic yellow vein virus* in a greenhouse trial. Moreover, no consistent cultivar-virus source interaction in the field could be detected.

For three years, several methodological questions have emerged with regard to the best way to sample a field and to have the best representation of the reality. The different questions were: How to avoid introducing bias into the analysis? What's the most real representation of the field when sampling? How can we analyze many samples and how can we reduce them staying representative? How much samples do we need? When must we collect the samples (beginning or end of culture)? How can we detect and identify the disease reliably and repeatedly? What's the diversity intra-beet? What's the degree of viral and vectorial heterogeneity of a soil? What's the spatial distribution of the virus (tetrads) in the plot (within plot)? What are the diversity of tetrads and the viral heterogeneity inter-plant inter-zone inter-plot of these tetrads? What's the viral tetrad diversity and heterogeneity intra- plant of such tetrads? What's the evolution of the infection, of the infectious potential and of the proportion of viruliferous vector (virus MPN divided by vector MPN) between sowing and harvesting? Are there other viruses associated with rhizomania (single, double or triple infection)? These questions are the methodological aspect of the analysis.
The substantive issue of the methodological questions consists to know how we can detect and identify the disease reliably and repeatedly. Firstly, the

virus (tetrads) distribution on the different rootlets of different beet (inter beet intra-zone) have been analysed. The viral distribution in the soil level was also analyzed to understand the infection dynamic in the field. To make sure that the heterogeneity isn't a bias issued from the technical analysis, the PCR of a root single sample were tested repeatedly. This part represents the second methodological question. A third methodological question: if we took a beet randomly in the field, is it representative of what we have in the field for each beet? Is there a zonation in the field or a virus/vectorial distribution? In order to assess the occurrence and content of B-pathotype and P-pathotype of BNYVV in mixed infection in one beet, a virus multiplex quantification by real-time reverse-tanscription polymerase chain reaction (qmRT-PCR) was developed, targeting BNYVV isolates from France. The primers were designed for quantification as the taqman probes. Another technical problem is to know which period to collect the samples. Is it better to collect in the beginning or in the end of the culture?

This section presents firstly complement information about the field assays studied in France and secondly, the different techniques used and developed in the thesis. Finaly, the problem of the sampling period is addressed as well as the heterogeneity in the tetrad results from the same sample.

A.3 Materials and methods

A.3.1 Agronomical information on the different fields

In 2009, the previous crop and the pre-previous crop for AL, BL, CL, DL, EL, FL, AI and BI are respectively spring barley and wheat, spring barley and wheat, durum wheat and wheat, durum wheat and sugar beet, durum wheat and unknown, durum wheat and wheat, wheat and winter barley, spring barley and wheat. The last crop of sugar beet for AL, BL, CL, DL, EL, FL, AI and BI is respectively two, two, two, two, two, two, four and eight years. The soil composition for AL, BL, CL, DL, EL, FL, AI and BI is respectively a clay-limestone, clay-limestone, silty clay, silty clay, clay, clay, clay-limestone and clay-limesotne soil.
In 2010, the previous crop and the pre-previous crop for AL, BL, CL, DL, EL, FL, GL, AI, BI and CI are respectively spring barley and wheat, onion and winter barley, spring barley and wheat, spring barley and durum wheat, wheat and maize, spring barley and durum wheat, spring barley and wheat, wheat and peas, wheat and maize, and unknown. The last crop of sugar beet for AL, BL, CL, DL, EL, FL, GL, AI, BI and CI is respectively three, three, twelve, three, four, three, unknown, four, eight and unknown years. The soil composition for AL, BL, CL, DL, EL, FL, GL, AI, BI and CI is respectively a clay-silt, clay-silt, silt, silty clay, clay-silt, silty clay, clay-silt, clay-silt, sandy clay and clay-silt soil.
In 2011, the previous crop and the pre-previous crop for AL, BL, CL, DL, EL, FL, GL, AI, BI and CI are respectively soft wheat and wheat, wheat and wheat, wheat and potato, wheat and onion, wheat and sugar beet, spring barley and durum wheat, spring barley and soft wheat, wheat and winter

barley, wheat and colza, and unknown. The last crop of sugar beet for AL, BL, CL, DL, EL, FL, GL, AI, BI and CI is respectively three, nine, four, three, two, three, six, four, four and unknown years. The soil composition for AL, BL, CL, DL, EL, FL, GL, AI, BI and CI is respectively a sandy clay-limestone, sandy clay-limestone, silty clay, silty clay, silty clay, limestone silty clay, sandy clay-limestone, clay-limestone, clay-limestone and unknown soil.

A.3.2 Root severity

The root severity was not measured in 2009 but well in 2010 and 2011. The protocol of roots notation is rather crude. The measure is not really accurate and the ITB estimated the root severity (%) by including various symptoms together in a notation as the proliferation of rootlets, the size of the root, the root constriction… The Table A.1 represents the notation given for foliar and root symptoms in May-June and September. The notation is given for the set of roots/rootlets (5-6) in one sample. And there were 50 samples analyzed to evaluate an average for the root severity in the zone of the field.

Table A.1 Foliar and root severity notation

	Foliar symptoms	Root symptoms
May-June	0 no symptom 1 symptom present	0 no symptom 1 symptom present : pinching or root necrosis
September	0 no symptom 1 foliar symptom visible 2 systemic symtpom	0 no symptom 1 medimum root symptom 2 strong root symptom

A.3.3 Heterogeneity in the tetrad results

The diversity already present inside the field and the evolution induced by the beet variety is not always easily distinguishable. Nevertheless, several varieties of beet in the same field have been analyzed to highlight a relationship between the virus pathotypes and the variety of beet. Three varieties were analyzed per field invistigated.
With regard to the substantive issue, we have searched the best procedure for a consistent viral analysis. When we took the root sample randomly in the beet root, with a single analyze, it resulted that we evidenced sometimes a mixed infection of different pathotypes in the same beet. The pathotype the most represented was called the “major tetrad” and the lowest represented was called the “minor tetrad”. We had various tetrads and various infections (simple or mixed) according to the variety. But, it seemed *a priori* that there was not clearly a specific tetrad related to a certain variety. After that, ten repetitions of analyze for each variety have been made. In the beginning, one sample analyzed was one composite sample of one root for each case. When we made ten repetitions, the result was never the same and there was a lot of variation in the response (Table A.2). The table shows the heterogeneity in the tetrad result of samples taken randomly. In many cases, the different tetrads/sequences occurred were mixed, but inconstantly

according to the different samples checked. What we can see also it's the higher percentage of presence of the tetrad when the variety is sick. This will be confirmed by the multiplex quantification. In response to such variations, the adoption of sample homogenization allowed to obtain systematical and repeatable result. In one sample, the root sample was the final root tip including the rootlets. We have homogenized the entire sample by lyophilisation. We have crushed the sample in a pestle and a mortar. After that, the result was systematically identical in the repetitions.

Table A.2. Heterogeneity in the tetrad results from ten repetitions for each variety analyzed in September 2009: inter-beet intra-zone diversity.

Sample number	Beet variety and region	Type of resistance	First result of tetrad	Percentage for ten repetitions					
				Simple AYHR	Simple SYHG	AYHR maj SYHG min	SYHG maj AYHR min	Negative	Other
A5	Bering sick (Ruan)	Rz1/T+ nematode	AYHR/SYHG	**70%**	0%	10%	20%	0%	0%
A6	Bering healthy (Ruan)	Rz1/T+ nematode	AYHR/SYHG	20%	**40%**	0%	0%	0%	40% ND
A10	Python sick (Montargis)	Rz1/T+ rhizo	AYHR/multiple	**100%**	0%	0%	0%	0%	0%
A11	Python healthy (Montargis)	Rz1/T+ rhizo	AYHR/AYHT	**70%**	0%	0%	0%	0%	10% AYHT & 20% none
A12	Britta (Yèvre)	Rz1Rz2/T++ rhizo	Simple SYHG	0%	**50%**	10%	0%	20%	20% ND
A17	Harmonia (Yèvre)	Rz1	SYHG/SYHR	20%	**70%**	0%	0%	10%	0%
A18	Encarta (Yèvre)	Rz1/susceptible	Simple SYHG	0%	**100%**	0%	0%	0%	0%
A23	Harmonia (Bondaroy)	Rz1	Simple AYHR	20%	**50%**	20%	0%	10%	0%
A24	Antoinetta (Bondaroy)	Rz1	SYHG/AYHR	0%	**60%**	10%	20%	10%	0%
A25	Débora (Bondaroy)	Rz1	Simple SYHG	0%	**80%**	0%	10%	0%	10% ND
A26	Ludwinia (Bondaroy)	Rz1Rz2/T++ rhizo	AYHR/SYHG	30%	**50%**	20%	0%	0%	0%
A28	Magistral (Bondaroy)	Rz1Rz2/T++ nematode	AYHR/SYHG	**60%**	30%	10%	0%	0%	0%
A33	Encarta (Bondaroy)	Rz1/susceptible	SYHG/AYHR	0%	**60%**	0%	40%	0%	0%

ND = Non determinated in sequencing ; AYHR/SYHG = AYHR in majority tetrad and SYHG in minority tetrad; T++ rhizo = Strong positive control of resistance to rhizomania; T+ rhizo = Positive control of resistance to rhizomania; Rz1 = Resistance gene; T+ nematode = Positive control of resistance to nematode

A.3.4 Lyophilization

With regard to the methodological-technological aspect, the lyophilization (freeze-dry) seems to be a reliable technique for the homogenization of samples. End of the taproot with rootlets were sampled. The entire sample was lyophilized and grounded with a mortar in a fine powder. This technique permits to analyze the sample repeatedly with the same constant answer. The best period for sampling seems to be around the harvest when the virus has had the time to multiply itself inside the beet variety. The use of RT-PCR is still appropriate to detect the rhizomania vector and virus. The RT-PCR gives also accurate results comparing to the ELISA. Duplex RT-PCR targeting BNYVV RNA3 and the vector and targeting BNYVV RNA3 and RNA5 were developed to gain time in the analysis of all the samples.

A.3.5 Nucleic acid extract

Extracts were taken from frozen samples. Total RNA was extracted from 100 mg of samples (roots) using the RNeasy plant mini kit (Qiagen). The samples were crushed using the Fastprep® system (Qbiogene, MP Biomedicals, Illkirch, France), following the manufacturer's instructions for

optimal RNA extraction from plants. The samples in 2010 were previously lyophilized and ground with pestle and mortar avoiding the step with the Fastprep® system. For the bioassay, nucleic acid was also extracted using a faster procedure that had been suggested by Thomson and Dietzgen (1995) and allowed the use of direct sap extracts. Then we evolved through the polysome extraction which gave better results. The RNAs were extracted from the whole mixed roots using the polysomes extraction protocol (Jupin *et al.*, 1990) followed by phenol extraction and ethanol precipitation of the RNAs.

A.3.6 Reverse transcription

For broad-spectrum detection, cDNA synthesis was performed in two steps. For First, 9.5 µl of reaction mix containing 0.5 µl of reverse primer, 9 µl of diethylpyrocarbonate (DEPC) treated water and 1 µl of RNA were incubated at 65 °C for 10 min. Second, a reaction mix composed of 4 µl of M-MLV RT buffer (Promega Corporation, Madison, WI, USA), 2 µl of dNTP (20 nmol), 0.25 µl of M-MLV Reverse Transcriptase (200 U/µl) (Promega Corporation, Madison, WI, USA) and 3.25 µl of DEPC treated water was added to the first reaction mix. The total volume (20 µl) was incubated at 42 °C for 60 min.

A.3.7 Primers and probes

A couple of primer BNYVV3(1)-forward (5'-CAGTTTATGATTTAGGGCACA-3') (sense : position 464 to 484) and BNYVV3(1)-reverse (5'-ATCATCATCAACACCGTCAG-3') (antisens: position 1098 to 1079) was used in 2009 and 2010 to amplify a p25-gene-containing portion (amplicon of 635 bp). The presence of RNA5 has been also analyzed for each sample. For this, the primer pair 5'-CATGAAGATTAAGACTATGAG-3' (sense: position 643 to 663) / 5'-CGTCACAAACGATCATCCAC-3' (antisens: 1171 to 1152) was used in 2009 and 2010 to amplify a p26-gene-containing portion (amplicon of 529 bp) of BNYVV RNA5. In 2010 and 2011, a duplex is used for the detection of RNA- 3 and RNA5. For the RNA 3, it is the same primers giving the 635 bp amplicon and for the RNA5, the new primer pair 5'-ATGTTTGTTGGTCCCCCGCT-3' (sense: position 915 to 934) / 5'-CGAGCCCGTAAACACCGCAT-3'(antisens: 1165 to 1146) was used to amplify a p26-gene-containing portion (amplicon of 251 bp).

In order to quantify the B-pathotype (tetrad AYHR) and the P-pathotype RNA3 (tetrad SYHG) of BNYVV, a RT-qmPCR was developed. The couple of primer Qtetradfor-forward (5'-TTCACTGGTGTATCACAATAAIACTAAACG-3') and Qtetradrev-reverse (5'-GAGGTAIAGAACATAACATTACTCTACATAAGG-3') was designed using the Beacon Designer 7.91 program (Premier Biosoft International) to amplify the region around the highly variable tetrad. For the multiplex quantification, AYHR-probe, a Taqman® probe (5'-TTTCGTGGTTTATTGTGTGCTTATCATAGGC-3') was developed, and was labelled at the 5'-end with the fluorescent reporter dye 6-FAM (6-carboxyfluorescein) and at the 3'-end with the BHQ-1 quencher. Another

Taqman® probe (5'-TTTCGTGGATTATTGTGTTCTTATCATGGGC-3'), SYHGb-probe was also developed for the multiplex and was labelled at the 5'-end with the fluorescent reporter dye Yakima Yellow and at the 3'-end with the BHQ-1 quencher. The probe and primers were provided by Eurogentec S.A. (Liège, Belgium).

The different primers and probes sequences used in the thesis are shown and gathered in appendix B.

A.3.8 RT-PCR detection

For RT-PCR detection, the cycling times and temperature were 94 °C for 2 min (1 cycle), 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s (35 cycles), 72 °C for 7 min (1 cycle) and finally conserved at 4 °C. The reaction component for each sample detected for RNA3 was 21 µl sterile water, 4 µl $MgCl_2$ (25 mM), 8 µl Green Gotaq® Flexi buffer (Promega Corporation, Madison, WI, USA), 1.2 µl dNTPs (20 nmol), 0.8 µl each primer (20 pmol), 0.2 µl Gotaq® polymerase (5 U/ µl) (Promega) and 6 µl cDNA. For the RNA5 detection, the reaction component for each sample was 13.12 µl sterile water, 2.5 µl $MgCl_2$ (25 mM), 5 µl Green Gotaq® Flexi buffer (Promega Corporation, Madison, WI, USA), 0.75 µl dNTPs (20 nmol), 0.5 µl each primer (20 pmol), 0.13 µl Gotaq® polymerase (5 U/ µl) (Promega) and 2.5 µl cDNA.
We made ten RT-PCR repetitions from a single sample from each varietal root. We confirmed that there was no bias coming from the RT-PCR technique. The result for the second question, the viability of the RT-PCR, was totally homogeneous.

A.3.9 Quantification

The real-time quantitative PCR was performed using the iCycler IQ™ Real-Time Detection system of Biorad (Hercules, CA) during the years 2009 to 2011 and on CFX96™ Real-Time System C1000 Touch™ Termal Cycler of Biorad during the year 2012. The RT-mqPCR in real time is based on the fluorescence change during the amplification process. The quantity of emitted fluorescence is directly proportional to the quantity of amplification product generated during the PCR reaction. The following steps for the RT-mqPCR conception and optimization were realized in the same way that the activity report from Meunier *et al.* (2004).

After alignment of different pathotypes of BNYVV, the multiplex detection of the tetrad AYHR for the B-pathotype and the tetrad SYHG for the P-pathotype have been chosen due to the fact that these two tetrads are the most common in the sugar beet in the region of Pithiviers. The primer pairs and the different probes have been elaborated with the help of the comprehensive and user-friendly program Beacon Designer 7.0 (Biorad) in the conserved region around the tetrad (RNA3 of the BNYVV). The primers Qtetradefor (position in RNA3: 588-616) and Qtetraderev (position in RNA3: 706-674) were used. The two taqman probes are the following: SYHGb probe and AYHR probe (position in RNA3: 626-655) were used. The design

of the primers and the probes were realized following the instructions proposed by the designer of the program Beacon Designer, by Poitras and Houde (2002) and by Marras (2006).

The choices of the fluorophores were made following the recommendations of Eurogentec for the double-dyes oligonucleotides for multiplexing detection. It is important to select dyes that give a good spectral separation to avoid overlap of the signal which is the case with Yakima Yellow and FAM (6-carboxy-fluorescine). The quencher BHQ-1^{TM} gives a lower background compared to TAMRA (6-carboxy-tetramethylrhodamine). Furthermore, it is a good cost effective alternative to VIC, and it can be detected in the same channel at the same wavelength, with no additional calibration needed. Consequently, the number of variables is low. The alignement of different BNYVV pathotypes is shown in the figure A.1. This alignment shows the position of the primers and probes used for the RT-mqPCR.

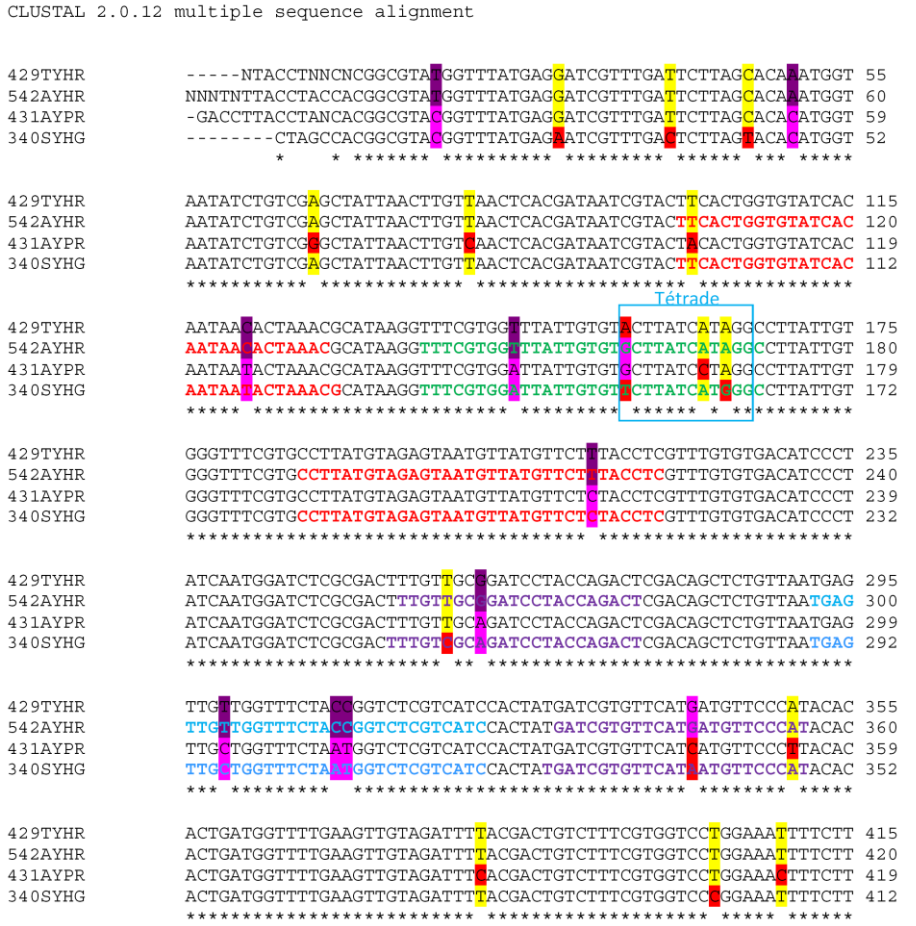

Fig. A.1. Alignment of BNYVV pathotypes for the design of primers and probes for the RT-mqPCR. In this figure, there are two propositions of taqman probe for the RT-mqPCR used to

distinguish the different pathotype. The alignment is made with four tetrads different from the three different pathotype encountered in the region of Pithiviers. The upper proposition has been used in the present study. And in a first step, only the detection for the tetrad AYHR and SYHG, which are the major tetrad encountered in the region, were used in duplex detection. The tetrad zone is shown in the box. The four primers are shown in red and the two probes in green between the primers. The nucleotide differences between the different pathotypes are also shown. The nucleotides marked in yellow are those that are found three times out of four; in red are those that are found one time out of four; in dark and clear purple those that are found two times out of four. The duplex RT-mqPCR detects here three SNP.

The primers were tested for optimization in classical RT-PCR and quantitative PCR with SYBR Green I (less specific due to the possible match with primer dimer) before going on with a quantitative PCR in real time with the new probe taqman. A melting curve was carried out to identify the possible dimers and/or the presence of several amplicons (through different fusion temperatures). Different concentrations in primers and probes were tested (50 nM-5µM). For the optimization in the concentration of primers and taqman probe, the primer concentration giving the weakest Cq and the highest ΔRn for a fixed quantity of cDNA is the optimal concentration to use for the RT-mqPCR. A concentration of 5 µM in primer and a concentration of 2,5 µM in probe seems optimal. The detection threshold for the qPCR in real time compared to classical RT-PCR and the repeatability were evaluated. The RNA extract was serial diluted before the RT-PCR. The RT-PCR detect the diluted sample at the 1000 X dilution and the RT-mqPCR in real time allow an effective detection for the 160 000 X dilution. With regard to the repeatability, several RT-mqPCR in real time were carried out independently to evaluate the variation due to the manipulations (minimal variation (1-4%) but cumulative due to the pipetting in each step). A solution to suppress these variations could be the possibility to carry out the reaction in a single step and also the use of intern standard.

The quantitative PCR with taqman probe was tested firstly in simplex and then in duplex. The taqman probe allows detecting three SNP in this study which procures a good specificity. The Master Mix from Eurogentec used was qPCR™ Master Mix RT-QP2X-03 (2x PCR Master Mix for probe assays with 5 mM final $MgCl_2$). False negative results due to PCR inhibitors could be excluded by using an internal amplification control in each PCR. To generate a regression line with a serially diluted solution, RNA standard were synthesized and tested in RT-mqPCR (Vaïanopoulos *et al.*, 2007). Two isolates were used, tetrad AYHR (B-pathotype) and tetrad SYHG (P-pathotype). The primers used were the same, Qtetradefor and Qtetraderev. After that, a cloning in a vector (pGEM®-T, Promega) was carried out in order to be stocked and multiplied. The presence of the sequence was verified by PCR with the primers before sequencing. The standard obtained were quantified by nanodrop (concentration by spectrophotometer: 274,2 ng/µl for SYHG clone or 68,8 x 10^{11} numbers of copy of SYHG clone; 297,3 ng/µl for AYHR clone or 74,4 x 10^{11} numbers of copy of AYHR clone; 90.5 ng/µl for ALS clone or 2,58 x 10^{10} numbers of copy of ALS clone) and adjusted in the same concentration before to be tested in RT-mqPCR with taqman probe and SYBR Green I. The clones are diluted 10 to 10 (10^{-1} -10^{-5}) in order to be used as standards for the qPCR reaction. The precision of the

dilution of the standards is important in order to obtain a good standard curve with PCR efficiency close to 100%. The Cq values are determined by RT-mqPCR with taqman probes targeting the targeted genes. The difference between the Cq values must stay constant and proportional to the dilution carried. A difference of 3,3 correspond to a dilution of 10. The two standards with the same concentrations should have the same Cq independently of the technique use (taqman or SYBR Green). It is the case for the use of SYBR Green but unfortunately it is not the case with the probes used in duplex which can be explained by the fluorophore pair compatibility. However, it could not be a difference in the kinetic reaction because it's an identical zone which was analyzed in all the isolates. Moreover, the RT-mqPCR in real time for the two standards must have the same efficiency. It's the case. Positive control and blank were also used to identify contaminations problems.

After that, an endogenous control was design in order to have an intern positive control (IPC) normalized and to avoid bias in the real-time RT-mqPCR (Huggett *et al.*, 2005). This allows an increase of the reliability of the results obtained through qPCR in eliminating the false-negative. The endogenous control is usually a gene of the host plant extracted in the same time that the total nucleic acid extracts of the plant. The endogenous control must have the same property than the virus targeted e.g. must be a RNA. This gene must be detectable by RT-PCR and it must have a unique amplicon (not two amplicons of different size due to the detection of mRNA and genomic DNA). Moreover, this gene must be easily detectable and have an expression level extremely constant in all the tissues tested and for all situation (infected plant and healthy plant (Nicot *et al*., 2005); young and older sugar beet and without regulation system; not affected by the experimental treatment; expressed in a similar level than the interest gene; and between all different varieties used) (Vandesompele *et al.*, 2002; Schmidlin *et al.*, 2008; Poitras and Houde, 2002; Konietzny and Greiner, 2003; Vaïanopoulos *et al.*, 2007; Mackay, 2002; Bustin *et al.*, 2009; Huggett *et al.*, 2005). The level of endogenous control must be also linked to the sample weight use before total RNA extraction. This level must be constant between all the different samples in order to make comparison. In view of the inherent variation in expression of housekeeping gene, it's recommended to use at least three proper stable control genes for calculating an accurate normalization factor of samples with relatively low expression variation.

Several endogeneous controls which should not fluctuate during the different cases were initially tested and selected according to the literature (Pfaffl *et al.*, 2004; Nico *et al.*, 2005; Nolan *et al.*, 2006; Saponari *et al.*, 2007; Gunnhild *et al.*, 2007; Hellemans *et al.*, 2007; Takle *et al*., 2007; Cepin *et al.*, 2010; Jarosova and Kundu, 2010; Mascia *et al.*, 2010; Hruz *et al.*, 2011). Different endogeneous controls in sugar beet were evaluated (gltutamine synthetase gene (gln2), acetolactate synthase gene (ALS), GAPDH gene, 18S riobosomal RNA gene) before the selection of the best endogeneous control used (Table A.3). Several sugar beet varieties (Sophia (*Rz1rz1*), Python (*Rz1rz1*), Ludwina (*Rz1rz1Rz2rz2*), Julietta (*Rz1rz1*), Bering

(*Rz1rz1*), Annoushka (*Rz1rz1*), Bison (*Rz1rz1*), Fiorenza (*Rz1rz1*), Adriana (*Rz1rz1*)) were tested by RT-PCR and RT-mqPCR (with sybergreen) with the different primers designed for each endogeneous control. The different endogeneous controls were also tested in an assay on healthy and sick (rhizomania) plants, on younger (1 month 1/2) and older (6 months) plants, on plants with different infection level and on plants infected with different viral isolates. The different quantitative assays were carried out with SYBR Green I to validate the detection before the taqman probe conception (with same annealing temperature than the BNYVV probe, fluorophore choice…). For some endogenous control, the primers designed were overlapping primers to avoid the amplification of genomic DNA.

The best results were obtained for ALS gene. The constance of the expression level of the endogenous control was verified (same Cq in each endogenous control for different samples tested (21,69 when tested with SYBR Green I). For each sample, the Cq value of the targeted gene (BNYVV RNA3) were transformed according to the Cq values of the ALS gene for the same sample. This gives the normalized Cq values of the sample. The RT reaction was made for RNA3 and ALS for the same sample in the same well. The catabolic 2-acetolactate synthase (ALS) seems to be a good endogenous control because this enzyme is required in the pyruvate metabolism after Calvin cycle of all plant cells. Indeed, ALS is required to transform two molecules of pyruvate in one molecule of 2-acetolactate and carbon dioxide. This last molecule is after that oxyded through chemical oxidation in diacetyl. ALS is responsible of the first step in the biosynthesis of branched-chain amino acids (valine, leucine and isoleucine). Theses three amino acids are essential amino acids. The different operons of the ALS are only regulated by feedback inhibition in the form of transcriptional attenuation (Tan and Medd, 2002; McCourt *et al.*, 2006; Chipman *et al.*, 1998; Joutel *et al.*, 1996; Dailey and Cronan, 1986). The sequence of the ALS in sugar beet has been provided by Heinz Himmelbauer (PhD in the Centre for Genomic Regulation (CRG) in Barcelona in Spain). This sequence is shown in the figure A.2. The probe and the primers used for the endogenous control are shown in this figure. The primers are the following: alsfor (5'- CTACCAACCTCGTATCAG -3'; position in ALS gene: 491-508) and alsrev (5'- GGCTTCCTTAACAATTCTAG -3'; position in ALS gene: 690-671). The taqman probe is the following: ALSgenrefprobe (Cy5-5'-TCTTGCTGACGCTCTCCTTGAT-3'-BHQ2) (position in ALS gene: 510-531). The design of the primers and the probes were realized following the instructions proposed by the designer of the program Beacon Designer 7.0 (Biorad), by Poitras and Houde (2002) and by Marras (2006). The choices of the fluorophores were made following the recommendations of Eurogentec for the double-dyes oligonucleotides for multiplexing detection (Triplex of probe and duplex of primers pair here) (Bertolini *et al.*, 2001; Bustin *et al.*, 2009). By using probes labeled with distinguishable reporter dyes, it is possible to run the target and reference amplifications in the same well.

Table A.3. Parameters of different endogenous control genes in sugar beet.

Accession Number/Name	Sequence Definition	Probe Sequence	GC%	Hairpin Bond(Internal)	Self Dimer dG(Internal)	Self Dimer Bond(Internal)	Pair Rating	Product Length	Product Tm	Product TaOpt	Cross-Dimer dG(Internal)	Cross-Dimer Bond(Internal)
AY026353	Beta vulgaris glutamine synthetase GS2 (gln2) mRNA, complete cds; nuclear gene for plastid product.	ATCCATTCCGTGGTGGTAACAAT	43,5	3	-1,6	3	81,8	83	72,1	53,3	-2,7	5
NM_114714	Arabidopsis thaliana acetolactate synthase (CSR1) mRNA, complete cds.	ACACTATCTAACAACGCATCGGCTA	44	0	0	0	85,8	101	77,7	57	-2,6	4
EF408234	gapdh mRNA	AGCCCTTCCACCTCTCCAGTCCTTAGC	59,3	0	0	0	79	150	79,8	61,4	-2	4
FJ669720	Beta vulgaris 18S ribosomal RNA gene, partial sequence.	TCGCTCCACCAACTAAGAACGG	54,5	0	0	0	89,6	106	77,5	57,3	0	3
	Sugar beet als from Heinz Himmelbauer	TCTTGCTGACGCTCTCCTTGAT	50	0	0	0	89,2	200	79,6	58,7	-0,2	3

	Sense Primer	Rating	Position	Length	Tm	GC%	GC Clamp	3'End dG	Self Dimer dG(Internal)	Hairpin dG(Internal)	Run/Repeat Length
gln2	CAGTGAAGTAATCTTATACCC	79,5	378	21	59,1	38,1	3	-4,3	-0,6	-0,6	3
als	ACCAGGTATCTGTATAGC	86,9	519	18	58,5	44,4	2	-3,6	-1,6	-1,2	2
gapdh	AAGGTTATCAACGACAAGTTTGGC	72,3	636	24	68,3	41,7	3	-5,2	-0,8	-0,8	3
18S	CCAGACATAGTAAGGATTGA	88,6	1.168	20	60	40	1	-3,1	0	0	2
als from Heinz Himmelbauer	CTACCAACCTCGTATCAG	88,7	572	18	59,9	50	1	-3,5	-0,3	-0,3	2

	Anti-sense Primer	Rating	Position	Length	Tm	GC%	GC Clamp	3'End dG	Self Dimer dG(Internal)	Hairpin dG(Internal)	Run/Repeat Length
gln2	ATGCGTCACATATTACCA	88,5	460	18	59,8	38,9	2	-3,8	-0,6	-0,6	2
als	CTGTGATTGCTACAAGAG	87,2	619	18	58,8	44,4	1	-3,5	-1,1	-1,1	2
gapdh	GGTGCTGCTGGGAATGATGTTAA	82,9	785	23	69,7	47,8	1	-2,7	0	0	3
18S	AACGGAATTAACCAGACA	88,2	1.273	18	59,9	38,9	1	-4	-1,2	0	2
als from Heinz Himmelbauer	GGCTTCCTTAACAATTCTAG	87,3	771	20	59,7	40	1	-3,1	-1,7	0	2

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catttcctcctcaaaagaacaagaacaagaacgagaacaagaacaaccatcctcattctctctccaaaa
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aattggcattgcggggtatgaataagattctggagtctagaatagggaagctgaatttggatttctccaagt
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cagtttatgaagttagtttgctgttgtcatgttacttgttactttaaaaagctttttgtagttttgagcaactagtatg
gaatgctcttcctgtattgcttggaaaattcacaaaagtggtttttcggctatggatgttgtgttgc

Fig. A.2. ALS sequence in sugar beet (provided by PhD Heinz Himmelbauer (CRG in Barcelona in Spain). The two primers, ALSfor and ALSrev are shown in red. The probe ALSgeneref is shown in green between the two primers.

The relative multiplex RT-mqPCR is an exponential process whereby the specifically amplified product ideally doubles each cycles. As such, the measured Cq value (standard name for threshold cycle or crossing point value according to the RDML guideless, http://www.rdml.org) is a logarithmic value that needs to be converted into a linear relative quantity using the following exponential function; $RQ_{unkn} = 2^{(Cq_{cal} - Cq_{unkn})}$ (Cq_{cal} is the Cq value of the calibrator sample (or reference sample, e.g. untreated control), C_{qunkn} the Cq value of the unknown sample, RQ_{unkn} the quantity of the unknown sample relative to the calibrator, and 2 the base of the exponential function, denoting 100% PCR efficiency) (Derveaux *et al.*, 2010). In any particular well, we know that the p25 reaction and the ALS reaction had exactly the same cDNA input. Therefore, it makes sense to calculate ΔCq

separately for each well repetition (double). The estimated error (standard deviation) is given as an asymmetric range of values, reflecting conversion of an exponential variable to a linear comparison. These ΔCq values can then be averaged before proceeding with the $2^{-\Delta Cq}$ calculation where ΔCq is the average of p25 Cq minus average of ALS Cq (Livak and Shmittgen, 2001).

The efficiency of the RT-mqPCR was accurate (good efficiency rate) when we did duplex detection of the two pathotypes (B- and P-pathotype) but it was not the case when we integrate the primers and probes for the endogenous control. Consequently, we tested separately the samples with the endogeneous control in another PCR reaction. On the one hand, duplex qPCR for the detection of the tetrad AYHR and SYHG was carried out with taqman probes and the other hand, the samples were tested for the ALS endogenous control gene in qPCR with SYBR Green. The samples tested by RT-mqPCR were efficient for the standard curve. The ALSgeneref probe seems run correctly only with the clones. Consequently, the gene ALS was tested by SYBR Green for 38 different samples. The efficiency of the standard curve was 93,8%, the R^2 equal to 99.2% and the melt curve presented a unique major peak. The constancy between the samples was good because the differences in Cq between these different samples were moslty weak. The Cq value was situated at 21,69 with a range of [21,69 - 1,51; 21,69 + 2,12].

If we normalized with the ALS gene (linearization) and showed the results in term of quantitative results Rq (ng/µl) rather than Cq, we should have had a better representation of the relative quantity of tetrad SYHG and AYHR in each variety (Figure A.3). However, the ALS reference gene was so expressed that the Cq values of ALS reference gene overrided the Cq values of the tetrads. The relative representation was not consequently clearly visible. If we wanted to know the relative level of ALS between each samples, we could calculate the proportion through 2exp(ΔCq endogenous gene). If we wanted to compare the BNYVV level in two samples, we had to use 2exp(ΔΔCq) where ΔΔCq is (Cq endogeneous gene in sample A minus Cq targeted gene in sample A) minus (Cq endogeneous gene in sample B minus Cq targeted gene in sample B). If we wanted to compare the relative level of each tetrad in a same sample, then it was simpler because it was the same Cq for the endogenous gene. To avoid the problem of surexpression of the endogeneous gene, we could also use Cqnormalized = Cqtetrad * (Cq mean ALS (of all samples)/Cq ALS(each sample).

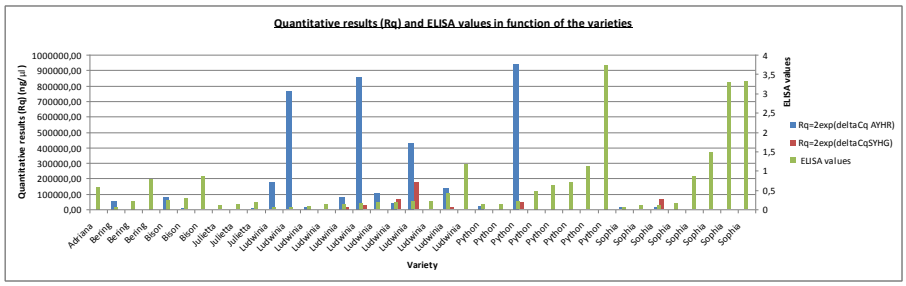

Fig. A.3. Correlation between the quantitative results (Rq) (ng/µl), the ELISA values and the sugar beet varieties used. The corrected optical density values (raw value minus two times the blank) are determined by DAS-ELISA targeting the CP protein coded by the BNYVV RNA2 of 38 samples from September 2010. The optical density is obtained by the mean of three absorbance values through the spectrophotometer. The values are corrected by subtraction of two times the value of negative control. The Rq is obtained by multiplex RT-mqPCR targeting the tetrad SYHG and AYHR in the RNA3. The Rq values are obtained by 2exp(deltaCqSYHG) and 2exp(deltaCqAYHR). Delta Cq is the difference of Cq between the Cq of the tetrads obtained by RT-mqPCR and the Cq of the ALS reference gene (by SYBRgreen) for the same sample.

In conclusion, the RT-mqPCR worked correctly to detect the tetrad AYHR and SYHG in duplex. The probes were able do discriminate the three acid nucleic difference between the two pathotypes focused in the analysis. A triplex RT-mqPCR targeting the A-pathotype could be also developed. However, we weren't able to use in triplex the probe targeting the ALS gene (endogeneous gene) in sugar beet. The Cq values for ALS were obtained by SYBR Green for each samples rather than taqman probe. The delta Cq was then calculated between the tetrad Cq values and the ALS Cq values. The relative quantity (Rq) of one tetrad compared with the other in each sample-variety (for the tetrad SYHG and AYHR) was calculated through 2exp(ΔCq for each tetrad) where ΔCq = CqAYHR - CqSYHG. To compare the sample between them with regard to the concentration of AYHR and SYHG, we use 2exp(ΔCq) where ΔCq = CqALS – Cqtetrad. This expression was used two times for each sample, firstly for AYHR and secondly for SYHG. Unfortunately, the ALS Cq was overexpressed comparing to the level of each tetrad. The Rq were consequently not really representative of what we have in each samples. Differences were masked by the endogenous gene. Even Cq is a exponential value, it was easier to see the influence of relative level of each tetrad through the Cq values than the Rq.

A.3.10 ELISA

Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) using the DSMZ immunological kit was carried out using polyclonal antibodies raised against BNYVV as described by Clark and Adams (1977) and by Koenig *et al*. (1997). The ELISA values are expressed in optical density (OD) or absorbance, extinction through the spectrophotometer. In spectrophotometry, it equal to 2 minus the log of the percentage transmittance of light. The degree of absorption of light or other radiant energy by a medium exposed to the energy is the absorbance. For solutions, it is the logarithm of the ratio of energy transmitted through pure solvent to

the energy transmitted through the solution. The DAS-ELISA was carried out on the samples coming from the different fields in May 2010, September 2010, May 2011 and September 2011. There were three repetitions. And the average value was measured as the average of the three repetitions value minus two times the blank value (not the negative control (uninfected plant) but only the ELISA solution without plant) for each batch (not for each plate) (Nahm and Goldblatt, 2004). This gives the corrected optical density (corrected OD).

Some problems with the detection by ELISA (antibodies) were encountered and several assays were carried out. Consequently, the ELISA detection and repetition for each sample were realized at different moment. Then, we obtained high standard deviation between the three repetitions for some samples. This was due to the potential degradation of the samples between each repetition, the change of ELISA products from the industry, the difference in the experimental manipulations, the change of new stock solutions... To take into account this deviations, the ELISA values were analyzed statistically.

A.3.11 Sequencing

The tetrads are identified by direct sequencing of the RNA3. Sequencing was carried out using the primer BNYVV3(1)-forward with the aim of assessing the region highly variable of the tetrad and to have a good portion of the p25 to see the different mutation positions. The sequencing was carried out using a Beckman Coulter CEQTEM 2000 SL (Beckam Coulter, Fullerton, CA). All the samples were sequenced with this primer. Sequences obtained were firstly checked in the NCBI online database software (http://www.ncbi.nlm.nih.gov) et after aligned with sequences already known using a multiple alignment editor program, Jalview 2.4 (Clamp *et al.*, 2004) and phylogenetic trees were conducted using MEGA version 4 (Tamura *et al.*, 2007). All acid nucleic sequences were also translated in aa with the online software program Expasy Proteomics Server (http://expasy.org/tools/).

A.3.12 Soil sample, soil preparation and serial dilution

To evaluate the infectious potential of a soil, a bioassay has been made. Two samples per year are made, one just after the seeding and the other one at the harvest. This bioassay consists in estimate the level of BNYVV and the level of *Polymyxa betae* in the soil. In summary, the purpose is to estimate the level of viruliferous *Polymyxa betae* in the soil. The estimation is based on the theory of “Most Probable Number” (MPN). The MPN gives us the probability for the numerical interpretation of the dilution data (Tuitert, 1990; Ciafardini, 1991). In 2009, eight soils (before the culture) have been analyzed; twenty soils (ten before the culture and ten after the harvest) in 2010 and also twenty in 2011. For each rhizomania-infested soil, the upper 25 cm of a field around Pithiviers was collected. The soil sample consists in homogenize a sample constituting 40 cores (1kg ground at all) by 25 cm

deep in each of four areas for each variety (3 varieties per field). Therefore, there are 12 samples per site. The soil was air-dried, mixed thoroughly and ground with pestle and mortar. Each soil is then sifted through a sieve of 2 mm in diameter. Samples of the infested soil were serially diluted with a dilution ratio of five and the number of dilutions required is seven. So the lowest dilution of a soil sample is 5^{-7}. Samples of the infested soil were serially diluted by volume with coarse sterilized dry sand. At every dilution step, the soil and sand were thoroughly mixed by vigorous shaking in an inflated plastic bag before collecting a composite sub-sample for the next dilution (Tuitert, 1990). Per dilution, the required hexagonal visotubes were filled with 35 ml of the mixture. In each visotube, a sensible sugar-beet seedling (cv. Cadyx (without *Rz1*), 2 days pre-germinated) was planted. The soil-sand samples were then humidified with a pipette containing a nutrient solution of Hoagland. The visotubes were placed in a greenhouse with a day and night temperature of 25 and 20 °C, respectively. The temperature of 25 °C is the optimum soil temperature for infection; the time between sowing and the first detectable infection is shortest and the subsequent rate of infection most rapid as this temperature. The photoperiod is sixteen hours (Blunt *et al.*, 1991; Webb *et al.*, 2000). To keep the soil moist, the Hoagland solution was regularly added in the visotube. The plants were analyzed for presence of *P. betae* and BNYVV (RNA3) after an incubation period of six weeks. For each zone of the twelve zones in a field, the presence of *P. betae* and BNYVV (tetrad) for each of the seven dilutions was measured by RT-PCR. The tetrads are also identified at different dilutions. So there is information about the dilution threshold where the virus and the vector were found. With those results, the MPN for the infective units per gram of soil of *P. betae* and the MPN for the infective units per gram of soil of the virus were calculated through the MPN calculator (developed by Parnow in 1972; http://www.i2workout.com/ mcuriale/mpn/index.html). To detect BNYVV in soil, the virus in Cadyx was firstly tested by RT-PCR because to have a beet infected by the virus, a germinated viruliferous *P. betae* infecting the beet must be present. Detection made here is more accurate because the RT-PCR technique rather than the ELISA were used and because the tetrad can also be given after sequencing. With those results, the infectious potential of the entire soil can be calculated because each zone corresponds to a repetition. There are consequently twelve repetitions per field and four repetitions per varietal band in 2010 and 2011. However, due to the lack of work time, in 2009, to reduce the quantity of work, three zones corresponding to the three bands of variety were mixed. So there were only four repetitions for the entire soil. To have the proportion of viruliferous vector (%), the virus MPN (IU/ gr soil) were divided by the vector MPN (IU/ gr soil). Consequently, we obtained the infective population of *P. betae* really viruliferous in a soil which has also germinated and migrated to the beet. In September, the viruliferous vector depends on the vector already present in the soil in May less the viruliferous vector which has germinated during the sugar beet season plus the new viruliferous vector added in the soil through the varieties.

The objectives of these bioassays are to permit the study of the spatial heterogeneity intra-varietal band and across the entire parcel. Another

objective is to study the evolution of infectious potential during a beet culture-period under the genetic constraint of the beet, during the beet intercultural period and also during the rotation. We searched also if there was a viral dynamic in the field. Firstly, the presence of a tetrad in a beet seems to be more related to the tetrad occurring in the soil. Indeed, we have analyzed the tetrad viral diversity and heterogeneity inter-plant inter-zone inter-field. It seems that there is not a clear answer for the pathotype of the virus in relation with the variety. After that, the resistance of the beet must certainly act and it's in the concentration of the virus that we can see if the resistance of the variety is good or not. This will be made in the quantification.
Finally, the objective of the study is to be conducted on at least three rotations. The total of sample for 2009, 2010 and 2011 represents more or less 4008 samples and 588 tetrads information. Consequently, for 2009 and 2010, there is 90 viral MPN information per band, 90 vector MPN information per band and 60 viral and MPN information per field.

A.3.13 Direct in situ hybridization

By direct *in situ* hybridization in the 3' end of all the five RNA's, we detect with a ssDNA wobble probe the presence of BNYVV in different tissues in the root sections of Tandem, Holly and HARRP transgenic-isogenic varieties (data not shown, UCL and SES Vanderhave in collaborative project, 2011). In the lab, it has been also showed by Diane Doucet *et al.* (2006) that the vector has a stronger relationship with the B-pathotype of the BNYVV than the A- or P-pathotype and that *P. betae* is localized in the peripherical tissues of the root. She has also showed by "in situ immunodetection reaction" that the P-pathotype virus is more localized in the root centre, near the phloem but not in the xylem. The concentration of the P-pathotype increases gradually from the epidermis until the phloem. For the A-pathotype, it's the same but the importance is lower than this one with the P-pathotype. However, the B-pathotype is more localized between the epidermis and the endoderm. These results can explain the heterogeneity distribution in the root and so the heterogeneity in the response from the ten repetitions in the analysis that we observed in the different samples.

APPENDIX B

Primers and probes sequences

Primer name	Sequence 5'-3'	Position	Amplicon size	Organism	RNA (Gene)	Th used
BNYVV2(1) for	acatttctatcctcctccac	804 to 823	545 bp	BNYVV	RNA2 (p75)	63 °C
BNYVV2(1) rev	accccaacaaactctctaac	1349 to 1330				
BSBV2for	cttacgctgttcacttttatgcc	2828 to 2850	399 bp	BSBV	RNA1	63 °C
BSBV2rev	gtccgcactcttttcaactgttc	3227 to 3205				
BVQ1(1)for	gctggagtatatcaccgatgac	2527 to 2547	291 bp	BVQ	RNA1	63 °C
BVQ1(1)rev	aaaatctcggatagcatccaac	2816 to 2796				
PB4for	caaacgcctgaaatcatctaac	811 to 831	170 bp	*Polymyxa betae*	etaeBS2	63 °C
PB4rev	gatggcccaattccttacac	970 to 951				
PSP1	tagacgcaggtcatcaacct	18S	520 bp	*Polymyxa betae*	ITS1	61 °C
PSP2rev	agggctctcgaaagcgcaa	5,8S				
BBSV(1)F	attagatcccacatcctggtgtggttaatc	3341 to 3370	304 bp	BBSV	3' end	58 °C
BBSV(1)R	gggcacctggaagaccaggtatataag	3644 to 3618				
BNYVV5(4)F	catgaagattaagactatgag	643 to 663	529 bp	BNYVV	RNA5 (p26)	58 °C
BNYVV5(4)R	cgtcacaaacgatcatccac	1171 to 1152				
BNYVV3(1)F	cagtttatgatttagggcaca	466 to 486	635 bp	BNYVV	RNA3 (p25)	58 °C
BNYVV3(1)R	atcatcatcaacaccgtcag	1100 to 1081				
BNYVV3P25for	tcggaatatacaaggtttaaaag	300 to 322	906 bp	BNYVV	RNA3 (p25)	53 °C
BNYVV3P25rev	gtcccaaccagatcaacaa	1205 to 1187				
BNYVV2P14(2)for	tatggggatggtagatagttt	4039 to 4059	385 bp	BNYVV	RNA2 (p14)	58 °C
BNYVV2P14(2)rev	ttacacctcaggatcgacaa	4423 to 4404				
BNYVV2P75for	ggtgagtttgaagggcttata	1357 to 1377	956 bp	BNYVV	RNA2 (p75)	56 °C
BNYVV2P75rev	ttttaccccgttccattta	2312 to 2294				
BNYVV5dfor	atgtttgttggtcccccgct	915 to 934	251 bp	BNYVV	RNA5 (p26)	58 °C
BNYVV5drev	cgagcccgtaaacaccgcat	1165 to 1146				
Qtetradefor	ttcactggtgtatcacaataa5actaaacg	588 to 616	119 bp	BNYVV	RNA3 (p25)	59 °C
Qtetraderev	gaggta5agaacataacattactctacataagg	706 to 674				
alsfor	ctaccaacctcgtatcag	491-508	200 bp	*Beta vulgaris*	(ALS)	59 °C
alsrev	ggcttccttaacaattctag	690-671				
BP14fSF	cgtatcgcctccctcgcgccat**cag**cgtgtctct**atgctaccaaagagcacgatg**	3851 to 3870	732 bp	BNYVV	RNA2 (p14)	66 °C
BP14fSR	ctatgcgccttgccagcccgc**tcag**ctcgcgtgtc**accacaacaaccttcggaac**	4512 to 4493				
BP25fSF	cgtatcgcctccctcgcgccat**cag**cgtgtctcta**ccgtcatgggtgatatattaggcgc**	314 to 338	722 bp	BNYVV	RNA3 (p25)	66 °C
BP25fSR	ctatgcgccttgccagcccgc**tcag**ctcgcgtgtc**tcaacaccgtcaggggcttga**	965 to 945				
BP26fSF	cgtatcgcctccctcgcgccat**cag**cgtgtctcta**atggcatatagcgacgataatcacc**	446 to 470	771 bp	BNYVV	RNA5 (p26)	66 °C
BP26fSR	ctatgcgccttgccagcccgc**tcag**ctcgcgtgtc**gcgggtagtttaacgtcttcggac**	1146 to 1123				
BP14fRz1F	cgtatcgcctccctcgcgccat**cag**tagtatcagc**tgctaccaaagagcacgatg**	3851 to 3870	732 bp	BNYVV	RNA2 (p14)	59 °C
BP14fRz1R	ctatgcgccttgccagcccgc**tcag**tctctatgcg**accacaacaaccttcggaac**	4512 to 4493				
BP25fRz1F	cgtatcgcctccctcgcgccat**cag**tagtatcagc**ccgtcatgggtgatatattaggcgc**	314 to 338	722 bp	BNYVV	RNA3 (p25)	66 °C
BP25fRz1R	ctatgcgccttgccagcccgc**tcag**tctctatgcg**tcaacaccgtcaggggcttga**	965 to 945				
BP26fRz1F	cgtatcgcctccctcgcgccat**cag**tagtatcagc**atggcatatagcgacgataatcacc**	446 to 470	771 bp	BNYVV	RNA5 (p26)	66 °C
BP26fRz1R	ctatgcgccttgccagcccgc**tcag**tctctatgcg**gcgggtagtttaacgtcttcggac**	1146 to 1123				
BP14fRz12F	cgtatcgcctccctcgcgccat**cag**tgatacgtct**tgctaccaaagagcacgatg**	3851 to 3870	732 bp	BNYVV	RNA2 (p14)	59 °C
BP14fRz12R	ctatgcgccttgccagcccgc**tcag**tactgagcta**accacaacaaccttcggaac**	4512 to 4493				
BP25fRz12F	cgtatcgcctccctcgcgccat**cag**tgatacgtct**ccgtcatgggtgatatattaggcgc**	314 to 338	722 bp	BNYVV	RNA3 (p25)	66 °C
BP25fRz12R	ctatgcgccttgccagcccgc**tcag**tactgagcta**tcaacaccgtcaggggcttga**	965 to 945				
BP26fRz12F	cgtatcgcctccctcgcgccat**cag**tgatacgtct**atggcatatagcgacgataatcacc**	446 to 470	771 bp	BNYVV	RNA5 (p26)	66 °C
BP26fRz12R	ctatgcgccttgccagcccgc**tcag**tactgagcta**gcgggtagtttaacgtcttcggac**	1146 to 1123				
Probe name	**Sequence 5'-3'**	**Position**	**Amplicon size**	**Organism**	**RNA (Gene)**	**Th used**
ALSgenrefprobe	Cy5-tcttgctgacgctctccttgat-BHQ2	510-531	/	*Beta vulgaris*	(ALS)	59 °C
Sonde SYHGb	YY-tttcgtggattattgtgttcttatcatgggc-BHQ1	626 to 655	/	BNYVV	RNA3 (p25)	59 °C
Sonde AYHR	FAM-tttcgtggtttattgtgtgcttatcataggc-BHQ1	626 to 655	/	BNYVV	RNA3 (p25)	59 °C