

Symptomology and yield impact of *pea necrotic yellow dwarf virus* (PNYDV) in faba bean (*Vicia faba L. minor*)

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Abstract We surveyed 33 symptomatic faba bean sites in central Germany towards the end of the growing season 2016 to analyse the suspected virus spectrum. All sites displayed plants with characteristic symptoms and had distinct funnel-shaped patches with a severely affected centre. The central core consisted of stunted, prematurely senescent plants. Symptomatic foci were scattered at random over a largely symptomless field. At two exemplary investigation sites we combined ground based yield assessments with remote sensing techniques to describe disease-loss relationships. Based on low altitude true-colour aerial imaging data, symptomatic patches were categorised into: (i) severely affected blackish core region, (ii) yellowish symptomatic

periphery, and (iii) a corresponding non-symptomatic reference patch. Serological tests revealed PNYDV (*Pea necrotic yellow dwarf virus*) together with PEMV (*Pea enation mosaic virus*) as dominant and equally abundant viruses. However, because PNYDV was significantly more restricted to the focal core than PEMV, we perceived PNYDV to be the causal agent for this apparently new symptom pattern in faba bean. As both viruses are vectored persistently by leguminous aphids, the observed symptom gradient within individual foci mirrored the epidemiological development over time, starting from an initial infection point and expanding towards the periphery via secondary virus spread to successively maturing and less susceptible plants. For each investigation site the segmented symptomatic surface of category (i) was 0.8 and 0.4%, for (ii) 20.5 and 6.4%, respectively. Combining the relative yield level for each symptom category with its respective surface, the overall yield gap at the field scale was extrapolated to 4.1 and 9.2% for grain yield and for 3.9 and 1.2% for crude protein. In the symptomatic core category, TKWs (thousand kernel weights) were halved due to enhanced proportions of shriveled grain. Because PNYDV-related yield decline was determined by the number, relative surface and disease intensity of individual foci, remote sensing techniques can offer valuable options for monitoring, loss assessment and agricultural decision-making.

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Introduction

In faba bean fungal diseases, weeds and insect pests are commonly regarded to be the most important biotic stress factors throughout Central Europe, usually dominating the relevance of the established virus spectrum transmitted by aphid vectors (Cockbain 1980, 1983; Lampkin 1994; Hoffmann and Schmutterer 1983). However, within the last decade virus surveys within several European states have detected and confirmed the wide spread establishment of *Pea necrotic yellow dwarf virus* (PNYDV) in commercial grain legume stands (Grigoras et al. 2010; Gaafar et al. 2016, 2017; Ziebell 2016, 2017). It is recognized as a new nanovirus species by the International Committee on Taxonomy of Viruses with a single-stranded, circular DNA genome comprising of eight genomic components (Vetten et al. 2012). Detailed genomic information and diagnostic tools became available to detect and identify specifically PNYDV (Grigoras et al. 2014; Gaafar et al. 2017). As other known nanovirus species, PNYDV is vectored by leguminous aphid species in a persistent manner (Vetten et al. 2012). However, epidemiological information is scarce and little is known about the PNYDV live cycle. The reasons for a growing PNYDV-status are currently under debate. Ziebell (2017) hypothesized an accumulation of anholocyclic hibernation of key vectors (e.g. *Acyrtosiphum pisum*) leading to an early dispersal of winged spring migrants and extended flight periods, thus virus dispersal (Froehlich 1962; Harrington et al. 2007; Habekuß et al. 2009; Hullé et al. 2010). An altered habitat structure with an increasing leguminous host density is a further suspected factor. Due to the effectual promotion of locally produced grain legumes by national and international agricultural programs, the grain legume cultivation area throughout Germany has almost doubled since 2013 (BMEL 2016a, b, p.9, FAO 2016).

According to current information, nanovirus symptom descriptions and virus related crop performance predominantly refer to single plants (Makkouk and Kumari 2009; Chen et al. 2011; Kraft and Pflieger 2001; Grigoras et al. 2010; Gaafar et al. 2017). For the first time, in 2016, massively abundant symptoms at the field level became apparent countrywide in Germany, mainly in fresh pea (*Pisum sativum*), faba bean (*Vicia faba*) and lentil (*Lens culinaris*), confirming the presence of PNYDV and associated viruses (Gaafar et al. 2016; Ziebell 2016, 2017). However, little is known

about the economic status of PNYDV infections at the field level.

Therefore, this study aimed to develop a first disease loss assessment scheme for symptomatic faba bean stands by linking ground-based viral infection status with yield parameters in related plant samples and adding the spatial component via remote sensing techniques. Low-altitude true colour aerial imaging with a relatively simple and cost efficient remote controlled hexacopter drone were chosen for measuring disease intensities with a distinct patchy appearance (Teng 1990; Jones 2004; Nutter et al. 2010; Sankaran et al. 2015).

The research objectives were: (1) to identify the virus spectrum involved, (2) to allocate infection status and corresponding yield parameters to distinct symptom categories, (3) to explore reproducible symptom-segmentation based on digital image information, and finally (4) to extrapolate symptom related yield losses to the field scale. The results aim to expand knowledge concerning the epidemiology and economic status of PNYDV as a new virus disease in grain legumes.

Materials & methods

The study consisted of two parts. The first part is a field survey appraising the general status and composition of viral infections in faba bean stands of the North Hesse area in 2016 (Fig. 1). The second part was a detailed analysis of two exemplary symptomatic sites within the same survey region and in the same year, employing digital aerial photography, virus diagnostics, and corresponding yield parameters for loss assessments (Fig. 1, site H and N).

Field survey 31 faba bean fields wloamy soils and a clay content ranging from 10 to 30% were sampled for virus incidence in the 2nd half of July 2016 where 10–50% of developed pods had reached their final length (growth stage code BBCH 70 to 75 according to Meier (2001), Table 1). The fields were located in a 15 × 30 km (450 km²) area representing almost 100% of all commercially grown faba beans in that area in 2016. Field management of all integrated sites was done according to good professional practice, including non-dressed seeds and herbicide treatments but no insecticides and fungicides were applied (Table 1, survey sites), except

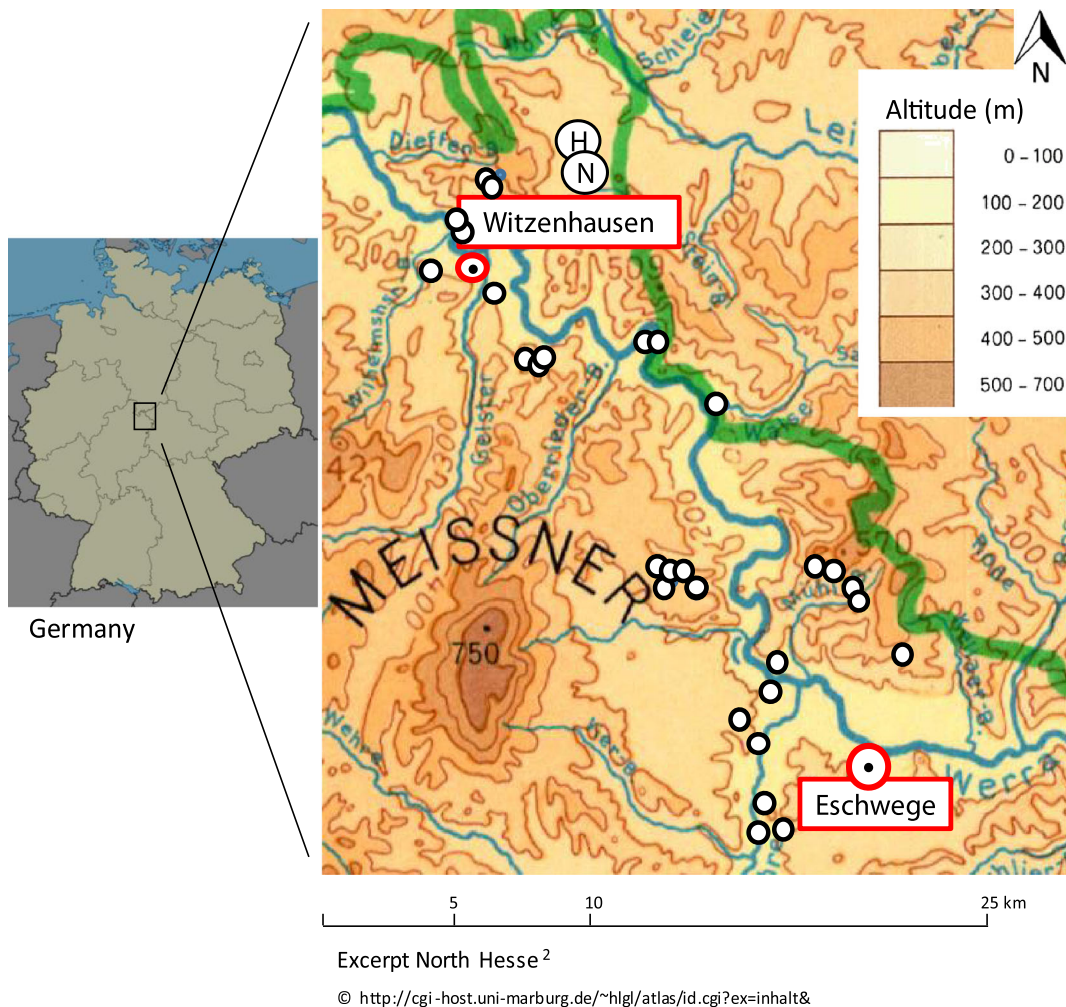


Fig. 1 Geographical distribution of the survey sites in North Hesse, Eschwege-Witzenhausen district, Germany. Black circles without numbering ($n = 31$) represent commercial faba bean sites.

Site ⊕ indicates the integrated commercial site near Friedland (seed propagation 'Fuego'), site ⊗ the organically managed faba bean plot at Neu-Eichenberg, 2016

one field receiving Pimiricarb once at the 25th April. The eight organic sites were weeded mechanically and there were no insecticidal interventions.

Field H, with a total field size of 24 ha, was located 2.3 km north of site N (Fig. 1) providing similar climatic and soil conditions. Here, variety 'Fuego' was sown on March 10 for seed propagation. Row spacing was similar to site N with a sowing density of about 40 kernels m^{-2} . Integrated management according to good professional practice included one herbicide and three insecticide treatments (λ -cyhalothrin targeting *Sitona lineatus* (Col.: Curculionidae) and aphids as potential virus vectors (Hom.: Aphididae) on May 10) and on June 3 and 30 for *Bruchus rufimanus* (Col.: Bruchidae).

The reported yield after post-harvest processing consisted of 0.438 $t ha^{-1}$ quality seeds and an additional fraction of 0.07 $t ha^{-1}$ low grade shrivelled and broken grains for animal feed (Table 1).

Field N, with a field size of 0.2 ha, was located at the organically managed experimental farm of the University of Kassel at Neu-Eichenberg (Germany) about 40 km NE from Kassel, 240 m above sea level, with 7.9 °C mean air temperature, 619 mm yearly precipitation and clay-silt soils on loess. The faba bean variety 'Fuego' was grown for animal feed with a row spacing of 15 cm and about 55 kernels m^{-2} (Table 1). Mechanical weeding was done once at May 11, no pesticides were applied. The harvested yield total was 0.299 $t ha^{-1}$.

Table 1 Details of on-farm investigation sites

	field survey	site H	site N
surveyed fields	31	1	1
management	8 organic, 23 integrated	integrated (seed propagation)	organic (animal feed)
Variety	acc. to practice	'Fuego'	'Fuego'
sowing date	acc. to practice	10.03.	10.04.
surface (ha)	acc. to practice	24	7
row spacing	acc. to practice	15 cm	15 cm
(seeds m ⁻²)	acc. to practice	40 m ⁻²	55 m ⁻²
seed dressing, fungicide treatments	–	–	–
herbicide treatments	yes, acc. to practice	yes, acc. to practice	none, mechanical
insecticide treatments	–	19.05.; 03.06.; 30.06. ^a	–
aerial digital photograph	–	22.07.	22.07.
number of sample replicates	1	19	7
per field			
position of sampling sites ^a	core ^b perimeter not sampled	reference ^b core 0.15 m ² perimeter ^b 0.15 m ²	core 0.15 m ² perimeter ^b 0.15 m ² reference 0.15 m ²
plant tissue sampled for later virus diagnostics	10 terminal leaves/site	basal stem cuts	basal stem cuts
virus incidence sampling	19.07.	31.07.	31.07.
yield parameter sampling	–	31.07.	31.07.
harvest on-farm	acc. to practice	10.08.	15.08.
yield, certified seed quality t * ha ^{-1 c}	–	0.44	n.d. ^d
yield, feed quality t * ha ^{-1 c}	–	0.07	0.299 ^d
yield total t * ha ^{-1 c}	–	0.51	0.299 ^d

^a λ-cyhalothrin applications targeting *Sitona lineatus* (Col.: Curculionidae), 19.05.; virus vectors (Hom.: Aphididae), 03.06.; *Bruchus ruffimanus* (Col.: Bruchidae), 30.06., respectively

^b symptomatic core (black), symptomatic perimeter (yellowish), non-symptomatic reference (green); for further explanations see text, [Materials and Methods](#) section

^c yield data based on 15% moisture content

^d n.d.: high quality fraction and small seed size fraction including broken and shrivelled grains were not differentiated

Acquisition of aerial photographs and preprocessing

True colour aerial images were taken from the exemplary sites H and N employing a commercially available remotely controlled hexacopter (Flightcopter.TV, Germany) equipped with a digital camera (Panasonic Lumix DMC-GF1). At both sites the hexacopter was flown the same day between 15:00 and 16:00 under overcast and dry weather conditions. Previously ground control points (GCPs) were acquired for the geo-referencing by using mobilemapper (Thales Navigation, Germany), ArcPad, version 7.0, and ArcGIS, version 9.1 (ESRI Geoinformatik GmbH, Germany). Six points were installed at the outer edges of site N, and a grid of six points placed at the edges and three points within the area of interest at site H. The digital pictures were transferred to a laptop computer, stored as JPEG files (Joint Photographic Experts Group). After inspection of the pictures for site H, a partial area of about 9.4 ha with largely homogeneous plant growth was subject to all further assessments. Areas with distorted plant growth due to abiotic stress and soil heterogeneity, were excluded from further analysis (Fig. 4). At site N the entire plot was analyzed. Individual images were georeferenced with ArcGIS 9.2 with reference to actual ground control points and converted into TIF format (Tagged Image File). After clipping, selected overlapping pictures were combined to one image (mosaicking) comprising the entire experimental area using the Mosaic Tool of Erdas Imagine 9.2 (Leica Geosystems GIS & Mapping LLC., Norcross, GA, USA), whereby histogram matching was applied for colour correction. The resulting pixel sizes had a resolution of 0.00991 m² and 0.00728 m² for the images of site H and N, respectively.

Segmentation

Both images were processed and analyzed using MAVI (Modular Algorithms for Volume Images, Fraunhofer ITWM, Kaiserslautern) implemented in a Framework called ToolIP, where numerous analytical solutions can be automatized by means of flow processing charts. The field borders in the images were masked out and a median filter was applied to smooth fine textures of canopy gaps with intermingled bare ground. The colour channels of the digital pictures were separated into red,

green and blue. The best visual match for the chlorotic area (Fig. 3c) was achieved by subtracting the blue from the red channel. The necrotic core area was best separated by subtracting the green from the red channel. In both cases the result was an 8-bit grey value image that could be segmented by means of the entropy analysis of the histogram (Kapur et al. 1985) achieving a global threshold with the interesting areas as foreground (Fig. 4, exemplar foci). The object-features-plugin of MAVI was used to measure the areas of each individual nest and their necrotic cores.

Plant sampling

Field survey For each field, paired plant samples were taken from a single symptomatic patch with typically chlorotic and stunted plants (see Results) in the centre on 19th of July at the ripening growth stage, BBCH 77 to 85. Terminal leaves from 10 individual main shoots were taken as a pooled sample within the stunted centre of infected foci and from a corresponding non-symptomatic green reference patch about 5–10 m away from the symptomatic core. Each leaf sample cohort was transferred into separate plastic bags for later virus diagnosis (Table 1). All samples were taken about 5–30 m away from the field edge.

Site H Based on the digital picture, 19 foci were selected in over the entire field (Fig. 4, H1), 5–250 m from the nearest field edge. Selection criteria were a largely regular shape, a clearly distinguishable blackish core with a yellowish perimeter, a clear separation from adjacent tractor lanes, and foci near to a non-symptomatic green zone. Furthermore, selected foci included small, mid-sized and larger foci. From each symptomatic focus, 0.15 m² square cuts were taken destructively about 2 weeks before expected harvest at BBCH 85. All primary and secondary shoots were cut directly above ground to obtain a succulent 1-cm basal stem cut, one per shoot. These stem cuts were pooled according to their focal position (core, perimeter and reference, Fig. 3c) and were transferred into separate plastic bags for later virus diagnosis (Table 1).

Site N According to the pre-selection criteria, all available, visually distinguishable foci ($n = 7$) were sampled. Virus diagnostics and yield assessments were done as described below for site H. All samples were taken about 1–8 m away from the field edge.

Virus etiology and maintenance

In 2009, a symptomatic pea sample was collected in Saxony-Anhalt, Germany. It was found to be infected with PNYDV as confirmed by serological profiling using different monoclonal antibodies in TAS-ELISA (Gaafar et al. 2016) and RCA-amplification and cloning and sequencing of all eight genomic components of PNYDV (Grigoras et al. 2010, 2014). Non-viruliferous aphids (*Acyrtosiphon pisum* and/or *Aphis fabae*) were allowed an acquisition access feeding period of 3 days on the symptomatic plant before being transferred to healthy plants (*Pisum sativum*, *Vicia faba*). After an inoculation access period of 3 days, aphids were killed using a pyrethrin based contact insecticide (Spruzit Schädlingfrei, Neudorff, Germany). Symptoms resembling those of the original pea sample occurred approximately 3–4 weeks post inoculation; symptoms of stunted growth, leaf curling and yellowing were also observed on faba bean plants and were similar to those in the field 2016, as described below (Fig. 2). These symptomatic plants were used for frequent serial passages of PNYDV to new target plants (Ziebell 2016).

Virus diagnostics

All samples were tested using DAS-ELISA for *Alfalfa mosaic virus*, *Cucumber mosaic virus*, *Pea enation mosaic virus* (PEMV), *Red clover vein mosaic virus*-like carlaviruses, luteo-/poleroviruses, potyviruses, and nanoviruses (Grigoras et al. 2014; Gaafar et al. 2017). Following the procedure described by Gaafar et al. (2017), PNYDV was detected by TAS-ELISA discriminating PNYDV against *Faba bean necrotic stunt virus* and *Faba bean necrotic yellows virus* (Abraham et al. 2012). Identity with GenBank Accession No. JN133279 was confirmed via PCR and PNYDV-specific primers (Gaafar et al. 2016).

Yield assessments

Yield data of the 19 sampled symptomatic foci at site H and seven at site N were obtained from the same sampling points and plant cohorts as for virus diagnosis. Whole plants preserved from 0.15 m² cuts at soil level were transferred into cardboard sacks and were kept for about 2 weeks at a dry and sheltered place to allow post-harvest ripening.

Fig. 2 Symptomatic group of faba bean plants (a), var. ‘Fuego’, with yellowish and elongated leaf tips at flowering stage (BBCH 65) and close-up of one of the terminal shoots (b). Individual leaf samples of all seven symptomatic plants (marked by arrows) were all serologically tested positive for both PNYDV- and PEMV-infection. Organically managed plot, University Kassel, Hessian State Domain Frankenhäusen, Grebenstein, 20.06.2016



Yield parameters were assessed for each fraction as the number of shoots m^{-2} , average number of pods and average kernel weight m^{-2} . Following harvest, seed samples were assessed for moisture content and thousand-kernel weight (TKW). The % raw protein content was determined for the harvested seeds of seven randomly selected foci from site H and all seven foci from site N with near-infrared spectroscopy (NIR) (Foss NIRSystem 6500, Silver Spring, Washington, USA and Microsoft tool for Windows by Infrasoft International, version 1.5, software package WIN ISI 1.5 2000; Tillmann et al. 2002). A Retsch Ultra-Centrifugal Mill ZM1000 (42,781 Haan, Germany) was utilized for preprocessing. All yields, TKW and percent protein values were standardized to 15% moisture (VDLUFA 1997; Wagner et al. 2007). To determine the yield impact of symptomatic patches for sites H and N, the respective sampling-point data (Table 3) were transferred into relative yields, with the factor 1.0 for the non-symptomatic reference, and were multiplied with the relative factors of the respective segment category (Table 4). The calculation of virus related yield deficit in tonnes ha^{-1} and the % relative yield gap was based on the extrapolated yield decline added to the reported on-farm yield to attain the expected potential yield of the hypothetically virus-unaffected crop stand (Table 4).

Statistical analysis

Statistical calculations were done with SAS v6.12 (SAS INSTITUTE Inc., 1989; SAS INSTITUTE Inc., 1990). All analyses of variance followed GLM procedures. Percentage values (e.g., protein concentration) were arcsin-square-root-transformed for statistical analysis. Untransformed means and transformed data (in brackets) are presented (Table 3). For the statistical analysis of virus incidence, contingency tables in SAS PROC FREQ McNemar-Test were used: here, for the 31 survey sites, one pair consisted of a symptomatic blackish core and its corresponding non-symptomatic reference. For sites H and N, two pairs of observations per field consisted of: i) a blackish core and its corresponding chlorotic perimeter, and ii) the chlorotic perimeter sample and its corresponding non-symptomatic reference.

Results

Symptoms

PNYDV was maintained under greenhouse conditions using aphids for frequent transfer to fresh target plants. Although originating from *P. sativum*, PNYDV was maintained on *V. faba*. The symptoms on *V. faba* resemble those observed on pea plants (stunted growth, leaf curling and yellowing, eventually death of plant) and clearly distinguish those plants from non-infected, healthy plants. Vector-mediated passages from *V. faba* back to *P. sativum* revealed identical symptoms. Identity of PNYDV was confirmed by TAS-ELISA (Gaafar et al. 2016), PCR using PNYDV-specific primer (Gaafar et al. 2017) and electron microscopy using purified particles (data not shown).

Virus-like symptoms in faba bean stands became obvious throughout North Hesse in June at flowering stage (BBCH 60–65) characterized by stunted growth, slight rolling, narrowing and elongation of leaflets with chlorotic leaf tips (Fig. 2). In the progressing season such yellowish patches expanded in size, later developing to the funnel-like appearance at ripening (BBCH 80–90, Fig. 3b). The centre region of symptomatic foci consisted of one or a small group of sterile, severely stunted and senescent plants with a prematurely blackish colour. A more or less distinct chlorotic ring encircled the blackish core regions, consisting of partially leaf shedding plants with premature leaflet abortion and a normal plant height (Fig. 3a and c). The yellowish and still succulent leaf-midribs remained attached to the stem.

At all survey and investigation sites H and N the distribution of symptomatic patches neither showed indications of a contagious distribution nor for a concentration at field edges (Fig. 4), with adjacent grassland, hedges or tree rows (not shown here).

Virus incidence

Field survey in North Hesse The two dominating viruses in all plant samples analysed in the North Hesse area ($N = 140$) were PNYDV and PEMV (Table 2). The spatial distribution of focal symptoms was similar to the sites H and N. All other viruses occurred sporadically, namely members of the Poleroviridae (BWYV 2-5G4) and Potyviridae family, respectively, with only two and four detections. *Alfalfa mosaic virus*, *Cucumber*

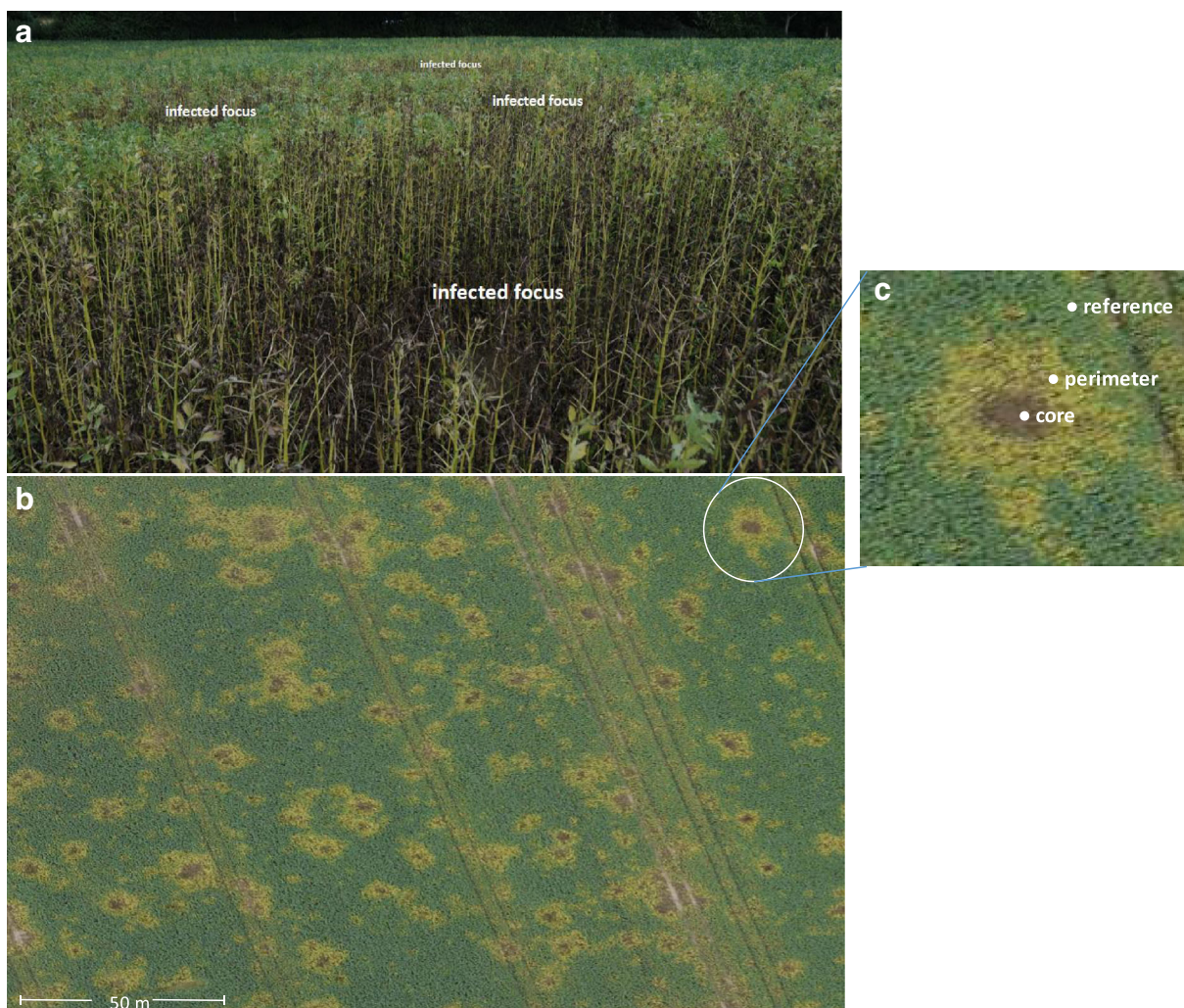


Fig. 3 **a** Symptomatic faba bean site H at BBCH 85, var. ‘Fuego’, with patches of stunted and premature blackish plants in the center. **b** Excerpt site H of an aerial photograph illustrating the focal

appearance of symptomatic areas and tractor lanes. **c** Close up of symptomatic focus. North Hesse, 22.07.2016

mosaic virus, Pea seed-borne virus or members of the genus Carlavirus were not detected.

In the symptomatic core, about 71% were PNYDV-positive plant samples which was significantly higher than in the non-symptomatic reference with 23% PNYDV-infected samples (Table 2). This pattern also applied to the detection of mixed PNYDV and PEMV infections, being significantly higher in the core-region than in the reference. In contrast, sole PEMV infections were detected in about 42–52% of the cases, regardless of the sampling position.

Investigation sites Similarly, at sites H and N the prevalence of single PNYDV and combined PNYDV-PEMV

infections were highest in the symptomatic core and chlorotic perimeter, dropping down sharply to 40% (site H) and 0% (site N) in the non-symptomatic periphery (Table 2). Site H only, there was a significant reduction of PEMV-incidence from the periphery to the non-symptomatic reference, whereas all samples at site N were PEMV-positive.

Areal images, yield extrapolation

Based on the processed true colour pictures, individual pixels were analysed in an area of 9.4 ha at site H and 0.2 ha at site N (Table 4). At site H and N pixel size varied with 0.00991 m² and 0.0073 m² (Table 1)

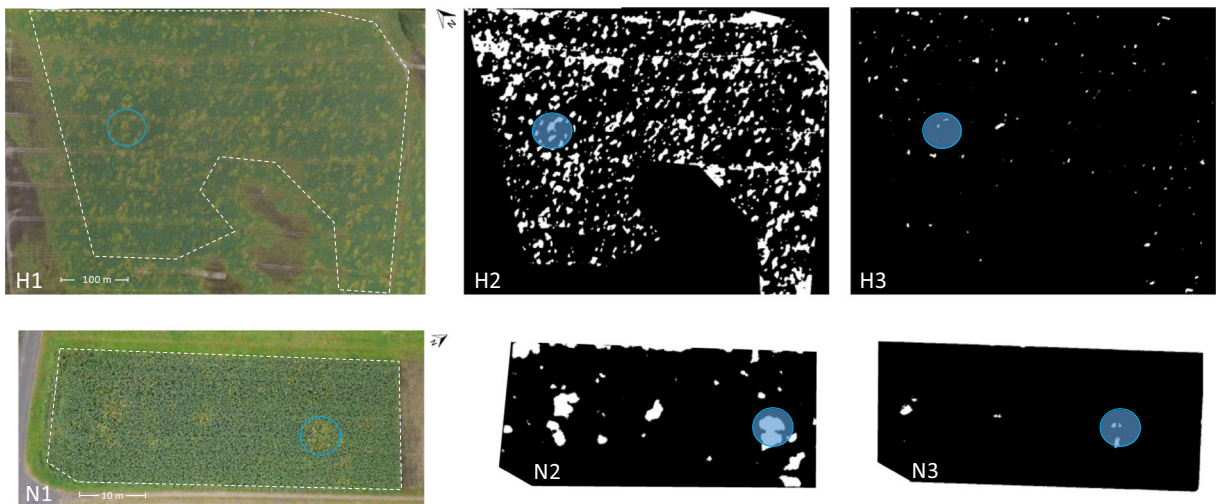


Fig. 4 Aerial photographs of faba bean fields H and N at 27.07.2017 and symptomatic areas of interest in two segmentation steps. H1 & N1: The broken white lines encircle the area subject for segmentation and excludes zones with known soil inhomogeneities at site H. White patches in H2 & N2 indicate

segmented symptomatic areas (yellow perimeter including blackish core). H3 & N3: White patches reflect the black core region within segmented foci. Blue circles: exemplary foci illustrating the segmentation steps 2), 3) for site H and N

resembling a calculated coverage of >0.5 pod-bearing shoots per pixel. The segmented relative surfaces for site H were about 0.8% severely affected “blackish core”, 20.5% symptomatic chlorotic perimeter, and 79% of the area symptomless. For site N the segmentation resulted in 0.4% symptomatic core, 6.4% yellowish periphery and 93% non-symptomatic area (Fig. 4, Table 4).

The density of individual symptomatic cores was about 70 and 127 foci ha^{-1} at sites H and N, respectively. Mean sizes of symptomatic patches (perimeter including blackish cores) ranged from 20.3 m^2 (Site H) to 1.8 m^2 (site N) and for blackish cores 1.1 m^2 and 0.3 m^2 , respectively (Table 4).

Yield parameters

Symptomatic plants displayed a significant reduction in pod setting compared to symptomless reference plants. However, the number of pods per shoot did not differ significantly within the chlorotic periphery and blackish core categories. Yields in the non-symptomatic reference patches exceeded 6 t ha^{-1} at both investigation sites and declined significantly to 54% (Site H) and 38% (Site N) in the symptomatic periphery and dropped significantly

further down to 20% and 13%, respectively, in the focal centre (Table 3).

A similar reduction pattern in the segmented categories applied to the kernel weight. In comparison to the reference patches, TKWs achieved just 70–80% in the chlorotic periphery and was almost halved in the symptomatic core (Table 3) due to smaller grains with a predominantly shrivelled, wrinkled appearance. At both investigation sites, symptom-related TKW decline was correlated with a reduced crude protein content (Fig. 5). There, 25% crude protein concentration and highest mean TKWs between 478 and 423 g dropped down to a concentration of 19% (site N) and 17% (site H) at TKWs below 259 and 232 g at site N and H, respectively (Table 2).

Overall, combining the area percentages of the three symptom categories with the corresponding relative yields (Table 4), the extrapolated yield reduction was 9% for harvested kernels and 3.9% for crude protein at site H. Site N presented a similar picture with 4.1% loss in kernel weight and 1.2% crude protein loss at field level.

Discussion

This study provides quantitative information concerning epidemiological traits and yield impact of a new virus

Table 2 Virus incidence of PNYDV and PEMV in *Vicia faba* plant samples obtained from symptomatic foci and corresponding non-symptomatic reference plants

surveyed fields/ analysed foci		df	PNYDV detections in %	Pr > S ^a	PEMV detections in %	Pr > S	mixed infection PNYDV & PEMV	Pr > S
31	symtomatic core	1	0.71	** ^b	0.42	ns	0.36	**
31	non-symptomatic reference	1	0.23		0.52		0.07	
site H analysed foci								
19	symtomatic core	1	1.0	n.a. ^c	0.8	ns	0.8	ns
19	symptomatic perimeter	1	1.0	** ^d	0.9	**	0.9	***
19	non-symptomatic reference	1	0.4		0.5		0.4	
site N analysed foci								
7	symtomatic core	1	1.0	* ^c	1.0	n.a.	0.4	(*)
7	symptomatic perimeter	1	0.6	(*) ^d	1.0	n.a.	0.2	(*)
7	non-symptomatic reference	1	0.0		1.0		0.0	

For sampling and diagnostic details see [Materials and Methods](#) section

^a Analysis of contingency tables as McNemar-Test comparisons and respective degree of freedom (df)

^b survey fields: comparison of symptomatic core versus non-symptomatic reference

^c comparison of symptomatic core versus symptomatic perimeter,

^d comparison of symptomatic perimeter versus non-symptomatic reference. (n.a.) analysis not applicable due to lacking variance (100% virus positive samples). (*) $P < 0.1$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

disease in commercial faba bean stands of central Germany in 2016.

Symptom development In all surveyed faba bean fields, symptomatic groups of plants at flowering stage had ceased stem elongation and displayed slight rolling and narrowing as well as yellowing of apical leaf tips (Fig. 2a and b). These symptoms resembled PNYDV-specific symptoms described previously for field pea (Grigoros et al. 2010; Gaafar et al. 2016), although premature blackening occurred exclusively in faba bean.

Virus spectrum PNYDV and PEMV were the most prominent viruses in all fields, regardless of whether they were managed integrated or organically (Table 1). Overall, PNYDV incidence was

most abundant within the symptomatic core (Table 2). In contrast to PEMV, its detection frequency diminished more sharply towards the perimeter and non-symptomatic reference areas. PEMV abundance was at times similar to PNYDV and occurred often as a mixed infections. However, PEMV was less restricted to the focal core fraction and often occurred in the periphery and unaffected reference areas (Table 2). Significant differences in the spatial distribution of PNYDV and PEMV and the absence of multiple infections with viruses other than PEMV lead to the conclusion that PNYDV was the only causal agent for the observed focal pattern, being independent from the presence of PEMV.

Table 3 Yield parameters at sites H and N of PNYDV and PEMV affected foci according to symptomatic zones blackish core, yellowish perimeter and non-symptomatic reference in faba beanobtained from 0.15 m² samples (for experimental details see [Materials and Methods](#) section)

	yield t*ha ⁻¹	TKW (g)	Pods * shoot ⁻¹	pod bearing shoots * m ⁻²	crude protein (% DM w/w) ^a	crude protein transf. ^b	crude protein t* ha ^{-1 a}
site H							
symtomatic core	1.30 ^a	232.7 ^a	6.5a	52.6 ^a	17.2	(0.43) ^a	0.30 ^a
symptomatic perimeter	3.50 ^b	297.3 ^b	10.0a	60.4 ^a	20.5	(0.47) ^b	0.65 ^b
non-symptomatic reference	6.54 ^c	423.3 ^c	11.6 b	57.2 ^a	25.1	(0.53) ^c	1.64 ^c
LSD ^c	0.87	27.2	1.77	8.5		0.025	0.35
Error d.f.	36	36	36	240.0		12	12
MSE ^d	1.20	1179.6	4.98	17.0		0.0003	0.06
Site N							
symtomatic core	0.79 ^a	259.2 ^a	3.9 ^a	67.6 ^a	19.7	(0.53) ^a	0.16 ^a
symptomatic perimeter	2.36 ^b	385.5 ^b	7.1 ^a	68.6 ^a	21.1	(0.48) ^b	0.51 ^b
non-symptomatic reference	6.21 ^c	478.3 ^c	11.8 ^b	70.5 ^a	24.8	(0.46) ^b	1.59 ^c
LSD	1.03	82.60	1.9	18.2		0.03	0.28
Error d.f.	12.00	12.00	12.00	80.0		12	12
MSE	0.52	3351.8	1.78	24.5		0.0004	0.04

^a Means of crude protein content in percent of dry matter (DM) and crude protein yield based on seven foci at site N. At site H seven foci were taken haphazardly out of the total 19 foci tested for virus incidence, yield, thousand-kernel weight (TKW), number of pods and shoots * m⁻².

^b Angle transformed percentages in paranthesis. Mean values in columns followed by different letters are significantly different at $p \leq 0.05$ (Tuckey test).

^c LSD least significant difference,

^d MSE mean square error

According to current information, such distinct nanovirus-related focal symptoms have not been described for faba beans at the field level, as other studies mostly refer to infected single plants (Makkouk and Kumari 2009; Kraft and Pflieger 2001; Grigoras et al. 2010; Gaafar et al. 2017). Thus, the focal appearance with a blackish core can be regarded as a PNYDV-specific feature for temperate faba beans when epidemics occur as in central Germany 2016; (Ziebell 2017; Lenz 2016; Krüssel 2017).

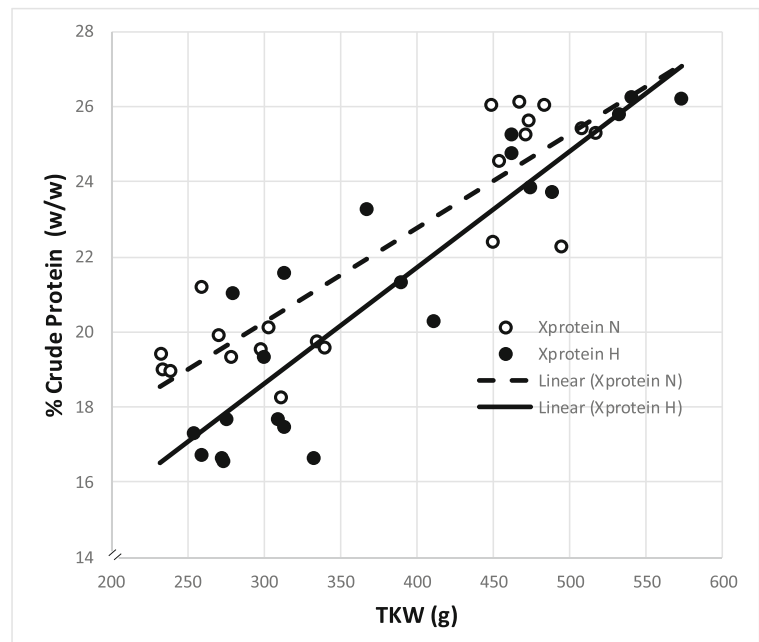
Epidemiology and vector interaction

Before interpreting the presented epidemiological findings, it is indispensable to refer to the established principle of persistent transmission (Sylvester 1989; Hogenhout et al. 2008; (Makkouk and Kumari 2009;

Brault et al. 2010) in drawing conclusions about the observed course of events. PNYDV is part of a three-cornered pathosystem (virus, vector and host), and is transmitted by several leguminous aphid species known also to transmit PEMV, namely *Acyrtosiphon pisum*, *Aphis fabae*, *Aphis craccivora* (Makkouk and Kumari 2009; Cockbain and Costa 1973; King et al. 2012), and further species under investigation (Ziebell 2017). For the persistently transmitted PEMV and Nanoviridae in general, other possible pathways, such as seed-borne-, leaf-, or root contact-transmission, as well as transmission by below-ground vectors, are not known and can be excluded.

Due to the visual behaviour in alate aphid vectors, host recognition depends on visible plant tissue above-ground (Moericke 1955; Johnson 1958; Döring and Chittka 2007). Thus, initial infections were set by winged aphid spring migrants. A single

Fig. 5 Linear regression of crude protein concentration and corresponding thousand-kernel weights of seven randomly selected foci, each focus comprising the three symptom categories blackish core, yellowish perimeter and reference at site H: $y = 0.311x + 9.27$, $R^2 = 0.817$; site N: $y = 0.025x + 12.73$, $R^2 = 0.789$



focus represents a single initial infection event (Hebblethwaite 1983; Madden et al. 2000; (Kraft and Pflieger 2001; Hebblethwaite 1983; Biddle et al. 2007; Makkouk and Kumari 2009). Expectedly, yield decline was most severe in the symptomatic core, when initial infections coincided with an early growth stage.

Subsequently, secondary virus spread was again vector driven by apterous nymphal or adult summer stages and focal expansion occurred with rapid horizontal transmission from plant to plant (Ehrhardt and Schmutterer 1964; Tsai et al. 1972). Factors affecting such transmission are physical and biological perturbations by wind, precipitation, intra-guild crowding, and predator and parasitoid activity as described for Barley yellow dwarf virus (BYDV), (Bailey et al. 1995; Smyrnioudis et al. 2007). In addition, integrated crop management involves the use of tractor lanes, contributing to mechanical disturbance due to regular tractor operations and sublethal insecticide exposure (Roberts et al. 1993; Perring et al. 1999). Therefore, the undirected displacement and resettling of infective apterous aphids is likely to cause the patchy, largely radial symptom expansion around a central initial infection point within an otherwise symptomless crop environment.

Other possible causal agents for symptomatic patches, namely aphid related damage by nutrient drain associated with primary and/or secondary fungal

infections, e.g. as described for BYDV in cereals by Greaves (1983), did not apply in the season 2016. Massive colonisation by aphids and relevant fungal diseases were negated by the local forecasting authorities throughout central Germany (Lenz 2016; Krüssel 2017) and was not observed in the regular visual inspections at all survey and investigation sites in North Hesse in 2016.

As the PNYDV-epidemic in 2016 developed at consistently inconspicuous resident aphid densities, it is clear that massive PNYDV-symptoms do not necessarily rely numerically on vector population sizes within the crop.

Areal photographs and segmentation accuracy

In the context of crop protection, remote sensing exploits altered reflectance properties of a crop, which are induced by either biotic stress causing agents and/or abiotic stress (Mahlein et al. 2012). Preferably, disease-specific spectral signatures support the identification of disease affected areas in general (Cui et al. 2009; Garcia and Barbedo 2017), although “specificity” as such, may be debatable (Bock et al. 2010). Using RGB digital photography, the pixel-information gathered represented reflectance mean values in cropping units of about 0.007 to 0.009 m² (Table 4). According to symptom category and the corresponding healthy and

Table 4 Segmentation of infection foci into the symptomatic classes blackish core, yellowish perimeter and non-symptomatic reference, summarized to land cover percentages and extrapolation of faba bean yield data from Table 3 into corresponding grain and protein yield gaps at investigation sites H and N

Picture analysis and segmentation									
	segment category	segmented area, m ² ^a	number segmented foci * ha ⁻¹	number segmented black cores * ha ⁻¹	mean size symptomatic foci m ²	mean size symptomatic core m ²	% segmented area		
Site H	symptomatic core	739		70.4	20.3	1.1	0.77		
	symptomatic perimeter	19,530	104.5				20.48		
	non-symptomatic reference	75,105					78.75		
	total	95,374					100		
Site N	symptomatic core	9		127.2	1.8	0.3	0.37		
	symptomatic perimeter	157	369.3				6.44		
	non-symptomatic reference	2,271					93.19		
	total	2,437					100		

Extrapolated yield parameters									
	relative yield factor to reference ^b	rel crude protein ratio to reference (Z)	yield gap in symptomatic segments t * ha ^c	% yield loss in segmented area ^c	yield gap crude protein in segmented area t * ha ⁻¹	% crude protein yield loss in segmented area ⁽⁴⁾			
Site H	0.20	0.68	-0.0032	0.60	-0.0003	0.24			
	0.54	0.82	-0.0484	8.60	-0.0048	3.63			
	1	1	0	0.00	0	0			
Site N	0.13	0.78	-0.0515	9.2	-0.00513	3.9			
	0.38	0.82	-0.0010	0.31	-0.0007	0.08			
	1	1	-0.0126	3.83	-0.0090	1.14			
			0	0.00	0	0			
			-0.0136	4.1	-0.0097	1.2			

^a segmentation based on the number of pixels with a size of 0.00991 m² (site H) and 0.00728 m² (site N)

^b relative yield decline in relation to non-symptomatic reference yields (=1.0), based on harvest data presented in Table 3

^c calculated yield gaps (t*ha⁻¹) and relative yield losses in percent are based on the reported on-farm yield at site H with 0.5085 and 0.315 t * ha⁻¹ at site N and by adding the extrapolated virus-related losses to an expected yield potential without virus incidence of 0.560- and 0.329 t*ha⁻¹ at site H, site N, respectively

diseased status, respectively, the spectral determinants were: a) crop coverage of erect plant parts, b) crop litter due to leaflet shedding, c) non-crop weeds, d) weed residues, and e) bare soil reflectance.

The digital images were taken on a single day towards the end of the cropping cycle. Its timing was a compromise between a sufficiently mature crop for yield assessment and distinguishable symptoms before the healthy plants developed natural senescent blackening. The obtained pattern mirrors a dynamic vector-driven epidemic development (see above). Inevitably, classifying faba bean stands into three symptomatic categories and selecting the best segmentation match of processed RGB-reflectance incidence, carries some subjectivity. Nevertheless, the two segmentation steps delineated the diseased area of interest and further, allocated a distinct blackish core fraction with reproducible results at two investigation sites employing the same faba bean variety with differing management (see encircled exemplary foci, Fig. 4, H2, H3, N2, N3).

Due to the relevance for correct symptom attribution, some partially deviating results at site H are discussed further:

Firstly, tractor lane delineation for the two symptom categories was predominantly sufficient (Fig. 4, H2, N2). The open lane space was omitted from sowing and herbicide treatment, and hence vegetation coverage was expectedly lowest in these spaces, facilitating their exclusion from both symptomatic categories. However, filamentous patterns along tractor lanes were allocated to the yellowish symptom category in some sections (Fig. 4, H1, H2). Suggested factors that may change reflectance and thus symptom allocations are an altered edge texture due to partial lodging of plants bending into the open lane space, irregular lane keeping during tractor operations, and thus variable lane width and/or patchy weed coverage. In those cases, a biased pixel allocation contributed to a slight overestimation in the presented mean size of individual foci and, conversely, underestimated their number due to thread-like connections (Table 4).

On the other hand, thread-like symptom allocation was likely to be accurate. The optical contrast between crop edge and bare soil is known to enhance the settling preference of visually-guided winged aphids (Moericke 1952; Johnson 1958; Kring 1972; Döring and Chittka 2007; Döring and Röhrig 2016). Therefore, concentrated settling behavior of viruliferous aphid spring migrants at

lane edges together with repeated physical perturbations of resident apterous vectors (see above) has to be taken into account for the observed aberrant symptom pattern along some tractor lane sections.

Lastly, the contours of the yellowish symptom category displayed a consistently elongated shape with a uniform east/west orientation (Fig. 4, H1, H2). Probably, directed vector displacement in the main west wind direction was involved, superimposed upon undirected radial secondary virus spread, leading finally to the asymmetric, “smeared” appearance of uniformly-oriented foci.

Overall, in order to distinguish between accurate symptom segmentation and segmentation artefacts, specific surveys will be required in future, covering the viral infection status in deviating segment areas to make adequate causal interpretations.

Yield impact We observed a close relationship between PNYDV abundance, symptom-intensity and a corresponding yield decline in grain weight and crude protein. However, this did not apply to all measured variables. For instance, the number of pod bearing main shoots m^{-2} was similar throughout the three symptom categories (Table 3) and even higher than the sowing density of 45–55 kernels m^{-2} (Table 1). At the first sight, this indicates secondary shoot formation, irrespective of the viral infection status. However, because our yield assessment scheme was restricted to pod-bearing shoots cut above-ground, sterile secondary shoots and entirely sterile individual plants were not considered. Thus, at this stage, it will require further investigation whether viral infections promote secondary branching.

Furthermore, a steady decline of seed weight as well as protein content occurred in a gradient from the reference area to the blackish core (Table 3). The correlation between an increasing shriveled grain fraction and a decreasing crude protein concentration (Fig. 5) may suggest virus related distortions in the pod-filling phase. Yet, which of the preceding steps from N-acquisition over protein synthesis and finally storage may be affected remains to be explored (Boulter 1981; Adler and Müntz 1983; Bailiss and Senayanyake 1984; Patrick and Stoddard 2010). Overall, the core fraction grain yields were reduced to less than 20% of the non-affected reference. Additionally, a halved TKW and

10–18% crude protein loss reflect the magnitude that PNYDV infections can have under field conditions, particularly when plants are infected in an early growth stage (Table 3).

Interpreting the extrapolated yield decline to the field level, the particular on-farm harvesting technique plays a crucial role and results may differ from our manually-harvested samples. Due to technical sifting effects, an unseen virus-related fraction of undersized kernels may have remained in the field and this would have lessened overall crop performance; therefore, yield loss may be underestimated. Furthermore, undersized but harvested grains with a lowered protein content lead to low-grade feed produce (Fig. 5), and, in seed propagation, undersized grains affect certifiable quality (Table 1).

Conclusions

Combining virus diagnostics, yield assessments and remote sensing techniques for spatial symptom analysis revealed the economic relevance of PNYDV in mixed infection with PEMV. However, an epidemic level of PNYDV shows this virus to be the causal agent for an apparently new focal symptom pattern in faba bean. Future research should develop appropriate monitoring and loss assessment techniques and address the life cycle determinants of PNYDV epidemics in faba bean and other leguminous crops.

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Compliance with ethical standards The authors declare their compliance with Ethical Standards.

Conflict of interest The authors declare that they have no conflict of interest.

Humans and animal studies This research did not involve experiments requiring a notification and permitting procedure with neither Humans and/or Animals nor genetic engineering research.

Informed consent Informed consent? yes, the authors agree with the EJPP procedure.

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