



Impact of *Pea necrotic yellow dwarf virus* (PNYDV) on nodulation, N₂ fixation and yield in faba bean (*Vicia faba*, L.)

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Abstract

Pea necrotic yellow dwarf virus (PNYDV) is a novel nanovirus in Europe, affecting various grain legumes. The impact of PNYDV on nodulation, symbiotic N₂ fixation and yield parameters in faba bean (*Vicia faba* L.) was investigated at controlled conditions in the greenhouse (2017), on-farm in natural infection foci (2018, 2019) and in a small-scale field experiment (2020). In the latter, the standard variety ‘Fuego’ was compared with the variety ‘GL Sunrise’ in early and late infections. In addition, the analogous effects for *Pea enation mosaic virus* (PEMV) were investigated under greenhouse conditions and the naturally occurring virus spectrum was recorded on-farm and in the field experiment. Results showed a much more severe impact of PNYDV than PEMV on nodulation, leghemoglobin status, N₂ fixation and finally yield, especially in early infections. Although ‘GL Sunrise’ was rated for a less symptomatic field performance toward PNYDV than ‘Fuego’, it showed a similar susceptibility in our field experiment where PNYDV was artificially inoculated to individual plants. Further research on the effect of plant varieties on susceptibility toward PNYDV infection and its spread in single or co-infection mainly with PEMV as a function of climate change acting upon their common aphid vector is required.

Keywords *Pea necrotic yellow dwarf virus* · *Pea enation mosaic virus* · *Vicia faba* · Nodulation · Symbiotic N₂ fixation · Yield

Introduction

Grain legumes can be affected by various virus diseases causing significant yield losses (Bos 2008). Among them, *Pea necrotic yellow dwarf virus* (PNYDV) infections (genus: *Nanovirus*, family: *Nanoviridae*) become increasingly important. Since its discovery in Saxony-Anhalt in 2009 (Grigoras et al. 2010), PNYDV is recognized as a new viral disease in several grain legumes and is now widespread in Central European countries (Gaafar et al. 2016, 2017, 2018; Grigoras et al. 2014; Ziebell 2017). A major severe PNYDV outbreak occurred in 2016 throughout Germany and Austria leading to significant yield losses (Gaafar et al.

2016). In their variety field trials in Gleisdorf, Austria, the breeding company “Saatzucht Gleisdorf GmbH” found that the variety ‘GL Sunrise’ performed better with regards to yield and obvious virus symptoms despite high PNYDV pressure; other varieties appeared to be more susceptible to PNYDV (more severe virus symptoms, higher disease occurrence, less yield). Thus, we included ‘GL Sunrise’ in the field infection experiment in addition to the widely used standard variety ‘Fuego’.

As all nanoviruses, PNYDV is phloem-restricted and transmitted by aphids in a circulative, non-propagative, persistent manner (Thomas et al. 2021) with *Acyrtosiphon pisum* as main vector (Ziebell 2017). Symptoms are described as stunted growth, yellow chlorotic leaves, leaf curling and subsequent top necrosis and plant death at later infection stages (Gaafar et al. 2016; Saucke et al. 2019; Ziebell 2017). In the field, symptomatic plants appear in form of individual foci, each representing an initial infection point caused by a winged spring migrant and spread by further aphids (Kumari and Makkouk 2007; Saucke et al. 2019). Mild winters caused by climate change promote anholocyclic overwintering and an earlier onset of spring

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flight of aphids in Europe (Habekuß et al. 2009; Harrington et al. 2007). In this context, virus outbreaks are expected to increase in the future (Canto et al. 2009).

Viruses have been found to affect nodulation (López et al. 2017; Taiwo et al. 2014; Tu and Ford 1984). Associated with rhizobia, legumes develop root nodules, in which the bacteria fix atmospheric nitrogen. Number and mass of nodules are correlated to plant performance (Allito et al. 2021). Inside the nodules, leghaemoglobin, causal for their red pigmentation, regulates the O₂ diffusion, whereby providing O₂ for respiration and at the same time protecting the O₂ sensitive nitrogenase (Wittenberg et al. 1974). For comparison of N₂ fixation capacity of different plants grown on the same soil, the natural ¹⁵N abundance method is an effective tool. It is based on the natural difference in the ¹⁴N to ¹⁵N isotope ratio between atmospheric nitrogen, which is globally uniform (Mariotti 1983), and other, organically transformed nitrogen sources (Shearer and Kohl 1986).

Little is known about PNYDV as a biotic stressor affecting plant growth and yield formation in protein crops such as faba bean. Because PNYDV often occurred in mixed infections with *Pea enation mosaic virus* (PEMV) (Saucke et al. 2019), PEMV has partially been included in this study.

This study focuses on the hypothesized impact of PNYDV on nodulation parameters, N₂ fixation and yield in *V. faba*. Three sets of experiments were conducted, in greenhouses with controlled PNYDV and PEMV infections and on-farm with naturally occurring PNYDV infection foci. In order to confirm and validate the previous findings, a small-scale field experiment was conducted that included a further faba bean variety with distinct symptomatic traits, covering early and late PNYDV infections according to plant development stage. The research objectives were (1) to assess whether PNYDV affects symbiotic N₂ fixation by characterizing its impact on nodule development, atmospheric nitrogen content in plant biomass and finally yield formation in comparison with PEMV; (2) to determine whether early and late infections differ in their impact on the above mentioned parameters and (3) to investigate if the reported low virus symptom field performance in variety ‘GL Sunrise’ is based on a lower susceptibility toward PNYDV in comparison with the standard variety ‘Fuego’.

Materials and methods

Virus inoculum

All virus acquisition and transmission experiments were carried out with the green pea aphid (*Acyrtosiphon pisum*, JKI clone) obtained from faba bean plants (‘Fuego’) infected with PNYDV, PEMV or virus free, respectively, received

from JKI Braunschweig. These aphid donor plants were cultivated in a greenhouse at 20 °C.

Experiment I: greenhouse, 2017

The impact of PNYDV and PEMV on nodulation and yield of *Vicia faba* cv. ‘Fuego’ was investigated in a greenhouse experiment (Table 1). Three plants per 4 l pot were cultivated in previously sieved arable soil obtained from a faba bean crop rotation (Neu-Eichenberg, North Hesse) at 16/10 °C day/night temperature at 16 h photoperiod. For virus acquisition, aphids were transferred from donor to experimental plants (13 aphids per plant) at BBCH 12 (two leaves unfolded), (Meier 2018). There were four replicates for each of the three variants: (1) PNYDV, (2) PEMV, (3) control (aphids without virus). The plants were caged with perforated crispac bags for one week of transmission feeding and afterwards treated with natural pyrethrines (“Schädlingsfrei CAREO Konzentrat”, 10 ml/l) to eliminate all vectors. Flowering plants were pollinated continuously with a paint brush until maturity.

Experiment II: on-farm investigation, 2018/2019

In 2018 and 2019, the nodulation status in PNYDV-affected *V. faba* cv. ‘Fuego’ at three different symptomatic levels was examined at agricultural fields in Northern Hesse, Germany (Table 1). The fields were managed according to common practice without the use of insecticides. A total of five PNYDV-infected foci, originated from three sites in 2018 and four foci from two sites in 2019, were sampled. The foci were divided visually into three fractions: (1) initially infected core, (2) secondary infected perimeter and (3) asymptomatic reference based on the severity of the symptoms (Saucke et al. 2019). At each sampling point, three representative plants were selected as pseudoreplicates.

Table 1 Experimental setup

	Experiment I	Experiment II	Experiment III
Variety	‘Fuego’	‘Fuego’	‘Fuego’ ‘GL Sunrise’
Inoculation	BBCH 12	Naturally	EI: BBCH 13 (early infection) LI: BBCH 62 (late infection)
Variants	PNYDV PEMV Control	Core Perimeter Reference	PNYDV Control
Nodule assessment	t1: BBCH 60-63 t2: BBCH 70-73	BBCH 72-76	BBCH 72-76
Harvest	BBCH 90	–	BBCH 90

Experiment III: small-scale field, 2020

A small-scale field experiment was carried out in order to validate the previously obtained results and to investigate the reports of a lower PNYDV symptom level in the variety ‘GL Sunrise’ (Table 1). The influence of PNYDV on nodulation and yield performance in early infections (EI) at BBCH 13 (three leaves unfolded) and late infections (LI) at BBCH 62 (flowers open two racemens per plant), respectively, in the varieties ‘Fuego’ and ‘GL Sunrise’ were investigated at the experimental farm site of the University of Kassel in Neu-Eichenberg (approximately 30 km NE from Kassel, 220–250 m above sea level, with 7.9 °C mean air temperature, 619 mm yearly precipitation and clay-silt soils on loess). These four variants (two varieties, two inoculation stages) were arranged with five replicates in a randomized block design in plots of 1.5 m × 6.0 m, 30 cm row width and a sowing density of 45 kernels m⁻². Each plot contained uninfected control plants as dependent samples. For inoculation, ten viruliferous *A. pisum* (carrying PNYDV) per plant were transferred to the field. To protect the aphids from possible interference by abiotic and biotic factors in the field, aphids were caged for three days in nylon fabric, carefully placed around the terminal shoot of faba bean seedlings. After removal, aphid propagation was terminated with pyrethrines applied with a hand sprayer (“Spruzit Schädlingsfrei,” 10 ml/l). In every plot, pseudoreplicates including two representatively grown plants for the virus-infected site and two corresponding plants from non-infected control site were assessed.

Virus detection by ELISA (see below) of the total 80 plants revealed 12 plants as PEMV positive and one plant as TuYV positive (Table 7). These 13 plants were removed from the data set. Thus, the variant of the early infected ‘Fuego’ has only four replicates instead of five and some samples have only one pseudoreplicate.

Nodule parameter assessment

In the greenhouse experiment, nodulation was assessed at two development stages: at onset of flowering (BBCH 60-63), hereinafter referred to as t1 and at the beginning of fruit development (BBCH 70-73) as t2. In the other experiments, on-farm and small-scale field, nodule assessment was carried out at the stage of fruit development (BBCH 72-76). Roots were carefully excavated 20 cm deep with their undisturbed root systems and cleaned with water. Lateral roots of field plants from experiments II and III were trimmed to a length of 2 cm apart from the vertical shoot axis, to assure uniformity and to exclude intermingled roots from neighboring plants. All nodules were plucked and counted.

For the investigation of nodule pigmentation, fresh nodules were dissected with a scalpel. In the following, two

different methods were used. Due to the high number of very small nodules in the greenhouse experiment, the ten largest nodules were selected and assigned to the pigmentation categories white or red (ranging from light rose to dark red). In the other experiments, 50 nodules were randomly selected to assign size and pigmentation status in the following categories: (1) ≤ 1 mm, (2) pure white, (3) red (light rose to dark red and minor green zones), (4) green (predominantly green nodules with a minor brown senescence zone) and (5) degrading (brown, often with a mushy consistency and/or damaged outer surface). Afterward all nodules were dried (2 days at 105 °C) and weighed. Nodule size was calculated by the quotient of weight and number of nodules.

N₂ fixation assessment

As indicators for N₂ fixation, the parameters nitrogen concentration and δ¹⁵N in above ground biomass were used in experiment II and in experiment III; additionally, the nitrogen derived from atmosphere (%Ndfa) was calculated. The latter could not be calculated for experiment II, as there was no corresponding reference plant here. Shoot length was measured and the above ground biomass dried (5 days at 60 °C). The analysis of nitrogen concentration and δ¹⁵N was carried out by the Centre for Stable Isotope Research and Analysis, University of Göttingen, using an elemental analyzer NA1110 (CE -Instruments, Rodano, Milano, Italy) and a mass spectrometer Delta XP (Thermo Electron, Bremen Germany) with a Conflo III interface (Werner et al. 1999). %Ndfa was calculated according to (Büchi et al. 2015) with a B value of -0.08, which was determined for ‘Fuego’. As a non-fixing reference plant served oat, variety ‘Max,’ sown manually in two 1 m² patches with 300 corn per m² at the same sowing date as faba bean. About 300 g aboveground biomass was taken from the center of each oat patch.

Yield assessment

Yield assessment was performed for the greenhouse experiment I and the small-scale field experiment III, the latter with two pseudoreplicates per plot. At maturity (BBCH 90), plants were harvested and the yield parameters number of pods, dry weight of kernels (5 days at 105 °C), TKW (thousand kernel weight) and nitrogen content in percent of dry kernel weight were determined. In experiment III, the number of shriveled grains (less than 0.5 cm in diameter) was additionally recorded.

Virus detection

In the outdoor experiments II and III, leaf samples were tested for presence of PNYDV, PEMV, TuYV and BLRV. In the on-farm investigation, pseudoreplicates were pooled

for each symptom category. Virus infection of plants was confirmed using ELISA (enzyme-linked immunosorbent assay). For detection of PNYDV and PEMV, Double Antibody Sandwich ELISA (DAS-ELISA) was carried out with polyclonal antibodies (coating antibodies and conjugate) produced at JKI as described previously (Fletcher et al. 2016; designated JKI-1604 and JKI-1841, respectively). Differentiation of TuYV and BLRV was done with Triple Antibody Sandwich ELISA (TAS-ELISA) using polyclonal coating antibodies (raised against beet western yellows virus, JKI-824 or BLRV, JKI-1817) and differentiating monoclonal antibodies (JKI-BLRV-2-5G4 and BLRV-4-6G4) as described previously (Abraham et al. 2006; Katul 1992).

Statistical analysis

Data were analyzed using R-4.0.4 (R Core Team 2017). All data were checked for normality of residuals and homogeneity of variance using Shapiro Wilk normality test and Levene test, respectively, as well as graphically with histograms, q–q plots and fitted values versus residuals.

For experiment I (greenhouse), analysis of variance (ANOVA) and Tukey HSD test ($\alpha=0.05$) were performed for t1 and t2 separately. The dataset of *nodule dry weight* at t2 was log-transformed to fulfill the assumptions.

The data from experiment II (on-farm) were separated in three fractions (core, perimeter and reference) as dependent samples of single foci. In 2018, there was a hierarchical structure of several foci in three sites, so that an analysis of significant differences was carried out using mixed effects models by nlme package (Pinheiro et al. 2021). *Fraction* was

the fixed effect and *focus*, *site* and their interactions, respectively were examined as random effects. The model with the best fitting random effect was selected by AIC and BIC. For post hoc test, emmeans was used (Lenth et al. 2019).

Experiment III (small-scale field) had paired samples of infected and control plants in respective parcels. Mixed effects models were used, with *variety*, *timepoint of inoculation* and their interactions as fixed effects and *parcel* as random effect. To fulfill the assumptions of mixed effects models, *nodule dry weight* was square-root-transformed and *dry weight per nodule* and *quantity share of shriveled kernels* were log-transformed. Post hoc test was conducted with emmeans.

Results

Experiment I: greenhouse, 2017

In the greenhouse experiment, *V. faba* plants of the variety ‘Fuego’ infected with PNYDV and PEMV, respectively, and non-infected control plants were examined for their nodulation at two different growth stages: at BBCH 60–63 (t1) and BBCH 70–73 (t2). Total number and dry weight of nodules per plant, as well as dry weight per nodule, were more affected at t1 than in t2 in all variants (Table 2). PNYDV infection had a significantly negative effect to number of nodules compared to the control, with a decrease of 92.3% at t1 and 88.8% at t2. The same applied to the dry weight with a decrease of 87.0% at t1 and 67.2% at t2. Dry weight per nodule was higher in PNYDV-infected than in control plants, albeit only significant at t2. PEMV-infected plants ranked

Table 2 Effect of virus infection and its increase or decrease compared to control on nodulation at BBCH 60–63 (t1) and BBCH 70–73 (t2) on *V. faba* ‘Fuego’ a greenhouse experiment

	t1			t2		
	PNYDV	PEMV	Control	PNYDV	PEMV	Control
Nodule number per plant	3.75 (2.17) c*	30.92 (9.26) b	48.42 (9.28) a	14.7 (10.5) b	112.8 (13.9) a	131.5 (38.0) a
in-/decrease (%)	↓ 92.3	↓ 36.1		↓ 88.8	↓ 14.2	
Nodule dry weight (mg) per plant	2.75 (1.85) b	10.83 (4.94) ab	21.08 (11.36) a	26.84 (16.11) c	56.13 (9.34) b	81.84 (6.87) a
in-/decrease (%)	↓ 87.0	↓ 48.6		↓ 67.2	↓ 31.4	
Dry weight (mg) per Nodule	0.83 (0.39) a	0.34 (0.07) b	0.42 (0.14) ab	2.36 (1.90) a	0.50 (0.11) b	0.66 (0.18) b
in-/decrease (%)	↑ 97.6	↓ 19.0		↑ 257.8	↓ 23.8	

Arithmetic mean and standard deviation in brackets

PNYDV pea necrotic yellow dwarf virus, PEMV pea enation mosaic virus

*Values with same letters in the same row per date (t1 & t2) are not significantly different at 5% level using Tukey HSD test

↑: increase

↓: decrease

between PNYDV and control in number and dry weight of nodules. They had significantly higher numbers of nodules at both times and nodule dry weights per plant at t2 compared to PNYDV. Compared to the control, PEMV nodule number was significantly lower at t1 with a decrease of 36.1%, but not at t2. Nodule weight was not significantly different at t1, but decreased significantly by 31.4% at t2. Dry weight per nodule was lowest in PEMV-infected plants, which was significant compared to PNYDV, but not compared to the control.

Dissection of nodules showed overall more red pigmented nodules at t2 than at t1 and more white pigmented nodules at t1 than t2 (Fig. 1). The amount of red nodule pigmentation in PNYDV-infected plants was lower than in the control. PEMV-infected plants had slightly fewer red nodules than the control, but much more than PNYDV-infected plants.

All four examined yield parameters were significantly reduced in PNYDV-infected plants compared to PEMV-infected and control plants (Table 3). The decrease was 96.0% for numbers of pods per plant, 100% in yield weight, 99.1% in TKW (thousand kernel weight) and 73.5 in nitrogen content as percent of dry matter. Values of PEMV-infected plants were very similar to the control values and not significantly different.

Experiment II: on-farm investigation, 2018/2019

In the on-farm investigation, naturally occurring foci with PNYDV symptoms were classified visually by severeness of symptom expression into core, perimeter and

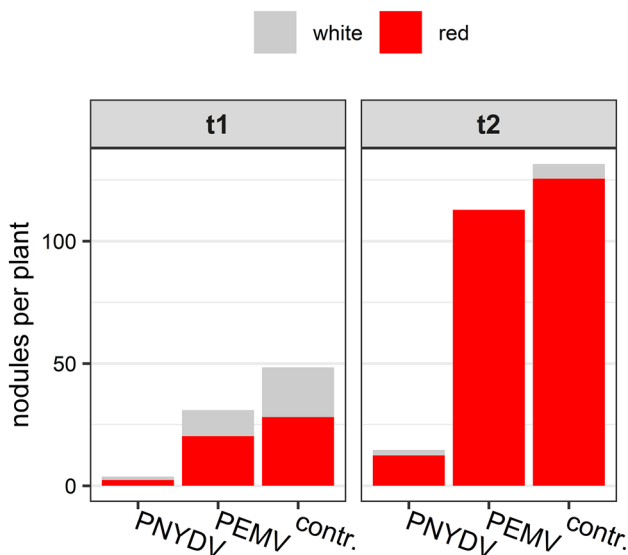


Fig. 1 Nodule pigmentation and number per plant of *V. faba* ‘Fuego’ at BBCH 60-63 (t1) and BBCH 70-73 (t2) after infection with PNYDV, PEMV and control, respectively, in a greenhouse experiment

Table 3 Effect of virus infection and its increase or decrease compared to control on yield parameter on *V. faba* ‘Fuego’ in a greenhouse experiment

	PNYDV	PEMV	Control
Pods per plant	0.08 (0.17) b*	2.04 (0.98) a	2.00 (0.33) a
in-/decrease (%)	↓ 96.0	↑ 2.0	
Yield per plant (g)	0.0 (0.01) b	3.14 (1.25) a	3.15 (0.67) a
in-/decrease (%)	↓ 100	↓ 0.3	
TKW (g)	5.00 (10.00) b	521.3 (41.99) a	526.4 (84.26) a
in-/decrease (%)	↓ 99.1	↓ 1.0	
N content (% DM)	1.10 (2.21) b	4.34 (0.44) a	4.15 (0.22) a
in-/decrease (%)	↓ 73.5	↑ 4.6	

Arithmetic mean and standard deviation in brackets

PNYDV pea necrotic yellow dwarf virus, PEMV pea enation mosaic virus

*Values with same letters in the same row are not significantly different at 5% level using Tukey HSD test

↑: increase

↓: decrease

(asymptomatic) reference. Nodule number and weight were significantly lower in the core, compared to perimeter and reference (Table 4). Nodule number decreased by 45.7% (2018) and 64.7% (2019) and nodule weight by 62.4% (2018) and 87.2% (2019) in the core when compared to the reference. The weight of single nodules was almost similar in core and perimeter in 2018 and decreased significantly by 33.8% and 30.5%, respectively. In 2019, weight per nodule within the core fraction decreased significantly by 66.7%, whereas nodules belonging to the perimeter were not significantly different from the reference.

In both growing seasons, 2018 and 2019, shoot length decreased significantly and gradually from reference to perimeter to core (Table 4), with an overall reduction down to 33.5% (2018) and 36.2% (2019) in the core. $\delta^{15}\text{N}$ values were significantly higher in core, than in reference, with a relative increase of about 81.3% (2018) and 145.8% (2019), respectively. In 2018, this value was highest in the perimeter fraction, but with a high standard error, therefore not significant. In 2019, the perimeter fraction revealed an interim position significantly distinguishable from core and reference. Nitrogen concentration in the shoots was highest in the references and decreased significantly in the core by 21.6% (2018) and 19.9% (2019), respectively.

Dissected nodules showed red pigmentation in the reference only (Fig. 2) with 23.5 (15.8, standard deviation) nodules per plant in 2018 and 24.2 (26.6) in 2019,

Table 4 Effect of PNYDV-infections and its increase or decrease compared to the reference plants on nodulation parameters, shoot length, $\delta^{15}\text{N}$ and nitrogen concentration in the shoot biomass of *V.*

faba ‘Fuego’ within naturally occurring foci in core-, perimeter- and reference fraction at on-farm sites in North Hesse in 2018 and 2019

	2018			2019		
	Core	Perimeter	Reference	Core	Perimeter	Reference
Nodule number per plant	58.8 (16.4) b*	122.8 (26.0) a	108.3 (17.4) a	45.8 (8.6) b	122.2 (13.1) a	129.7 (4.3) a
in-/decrease (%)	↓ 45.7	↑ 13.4		↓ 64.7	↓ 5.8	
Nodule dry weight per plant (mg)	60.8 (30.2) b	131.7 (31.1) a	161.6 (28.2) a	31.2 (9.19) b	192.2 (46.0) a	244.3 (28.7) a
in-/decrease (%)	↓ 62.4	↓ 18.5		↓ 87.2	↓ 21.3	
Dry weight per Nodule (mg)	1.00 (0.07) b	1.05 (0.13) b	1.51 (0.13) a	0.63 (0.09) b	1.46 (0.25) a	1.89 (0.22) a
in-/decrease (%)	↓ 33.8	↓ 30.5		↓ 66.7	↓ 22.8	
Shoot length (cm)	65.1 (6.91) c	82.3 (7.24) b	97.9 (7.83) a	74.1 (9.69) c	102.4 (7.75) b	116.1 (7.78) a
in-/decrease (%)	↓ 33.5	↓ 15.9		↓ 36.2	↓ 11.8	
$\delta^{15}\text{N}$ (‰)	2.03 (0.21) a	2.41 (0.84) ab	1.12 (0.09) b	2.90 (0.36) a	2.18 (0.39) b	1.18 (0.49) c
in-/decrease (%)	↑ 81.3	↑ 115.2		↑ 145.8	↑ 84.7	
N in shoot (%)	2.21 (0.11) b	2.11 (0.12) b	2.82 (0.15) a	1.97 (0.03) b	2.29 (0.02) a	2.46 (0.16) a
in-/decrease (%)	↓ 21.6	↓ 25.2		↓ 19.9	↓ 6.9	

Adjusted mean and standard error in brackets calculated with emmeans

*Values with same letters in the same row per year are not significantly different at 5% level using emmeans

↑: increase

↓: decrease

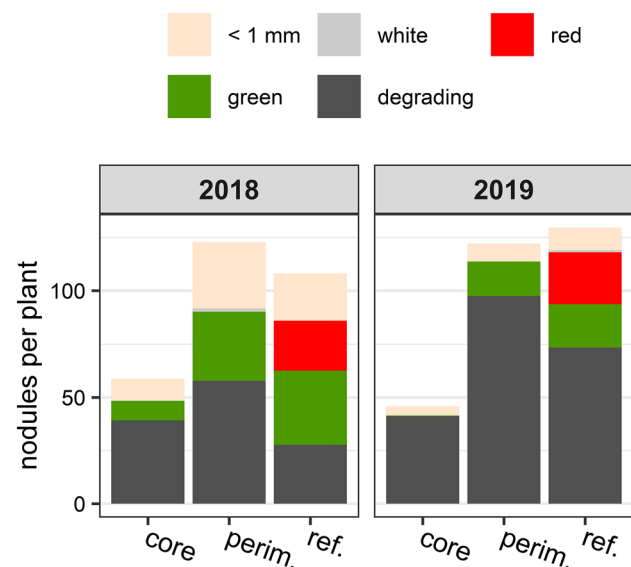


Fig. 2 Nodule pigmentation and number per plant of *V. faba* ‘Fuego’ in two growing seasons (2018 and 2019) in core, perimeter and reference of naturally occurred foci in an on-farm investigation

respectively. Plants from the core had less green and small nodules than those from perimeter or reference. Degrading

nodules were predominantly observed in the perimeter fraction.

Experiment III: small-scale field, 2020

In the experimental variety comparison, two *V. faba* varieties ‘Fuego’ (-F) and ‘GL Sunrise’ (-S) were inoculated with PNYDV at an early (EI) and late (LI) developmental stage, respectively. Uninfected plants served as control (C). For both varieties, EI resulted in a significant decrease in the three examined nodule parameters (Table 5). In number of nodules, this decrease was 49.3% for EI-F and 73.6% for EI-S; in nodule weight, it was 81.2% for EI-F and 93.1% for EI-S and in weight per nodule, it was 65.7% for EI-F and 74.1% for EI-S. EI-S had consistently lower values than EI-F, although not significant. Nodulation parameters for LI had an interim position in both varieties between EI and C, whereby values of ‘GL Sunrise’ were comparatively higher than those of ‘Fuego’. In most cases, LI differed significantly from EI, excluding number of nodules and dry weight per nodule in ‘Fuego’. LI did not differ significantly from C.

$\delta^{15}\text{N}$ values decreased gradually from EI to LI to C (Table 5). For ‘GL Sunrise’, the magnitude was larger and significant only between EI-S and both controls as well as

Table 5 Effect of PNYDV-infection and its increase or decrease compared to control on nodulation, $\delta^{15}\text{N}$ and nitrogen concentration in the shoot on two *V. faba* varieties ‘Fuego’ and ‘GL Sunrise’ after early (BBCH 13) and late (BBCH 62) infection

	‘Fuego’			‘GL Sunrise’		
	EI	LI	C	EI	LI	C
Nodule number per plant	91.9 (9.35) bc*	148.8 (17.4) ab	181.1 (21.6) a	54.5 (15.0) c	206.2 (22.9) a	206.6 (21.16) a
in-/decrease (%)	↓ 49.3	↓ 17.8		↓ 73.6	↓ 0.2	
Nodule dry weight per plant (mg)	51.5 (13.9) b	179.7 (37.1) a	273.9 (27.1) a	24.2 (6.6) b	255.0 (33.7) a	348.7 (37.43) a
in-/decrease (%)	↓ 81.2	↓ 34.4		↓ 93.1	↓ 26.9	
Dry weight per Nodule (mg)	0.56 (0.11) bc	1.20 (0.16) ab	1.63 (0.21) a	0.46 (0.09) c	1.27 (0.11) a	2.77 (0.16) a
in-/decrease (%)	↓ 65.7	↓ 26.0		↓ 74.1	↓ 28.4	
$\delta^{15}\text{N}$ (‰)	1.98 (0.23) ab	1.67 (0.18) b	1.61 (0.06) b	2.52 (0.15) a	2.12 (0.20) ab	1.52 (0.12) b
in-/decrease (%)	↑ 23.0	↑ 3.7		↑ 65.8	↑ 39.5	
%Ndfa	34.7 (7.31) ab	44.5 (5.55) a	46.5 (1.72) a	17.6 (4.66) b	30.4 (6.22) ab	49.2 (3.89) a
in-/decrease (%)	↓ 25.4	↓ 4.3		↓ 64.2	↓ 38.2	
N in shoot (%)	2.44 (0.31) ab	2.90 (0.23) ab	3.40 (0.05) a	2.30 (0.07) b	2.66 (0.12) b	3.30 (0.08) a
in-/decrease (%)	↓ 28.2	↓ 14.7		↓ 30.3	↓ 19.4	

Adjusted mean and standard error in brackets calculated with emmeans

EI early infection, LI late infection, C control

*Values with same letters in the same row are not significantly different at 5% level using emmeans

↑: increase

↓: decrease

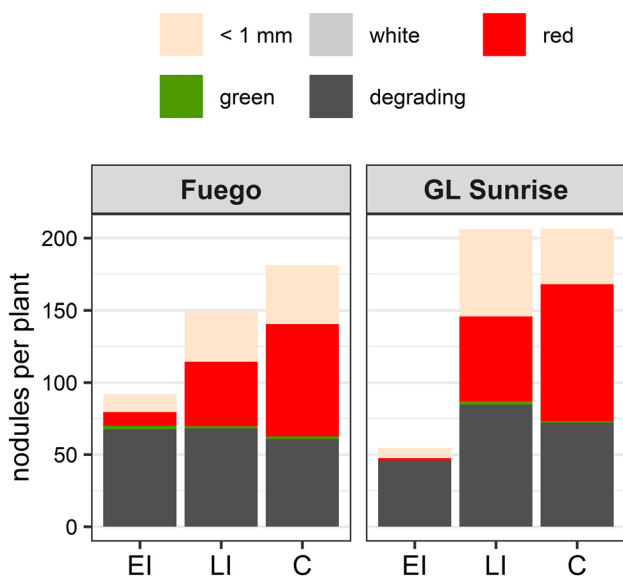


Fig. 3 Nodule pigmentation and number per plant of two *V. faba* varieties ‘Fuego’ and ‘GL Sunrise’ after early (EI at BBCH 13) and late (LI at BBCH 62) primary infection in a small-scale field experiment

LI-F. Employing oat as a reference plant with a $\delta^{15}\text{N}$ of 3.078, the %Ndfa corresponded vice versa to $\delta^{15}\text{N}$ and

decreased significantly by 64.2% in EI-S. Nitrogen concentration in shoots was consistently higher in controls. Its non-significant decrease for ‘Fuego’ was 28.2% (EI) and 14.7% (LI). In ‘GL Sunrise’, it decreased significantly by 30.3% (EI) and 19.4% (LI).

In all variants, there was a large proportion of degrading nodules (Fig. 3). Nodules of EI-S hardly had any red pigmentation, while those of EI-F had at least 9.5 (12.9) per plant. Late infected plants had 44.8 (28.4), (LI-F) and 58.8 (27.7), (LI-S) red nodules. Control plants had the highest number of red nodules per plant, 78.0 (28.2), (C-F) and 94.8 (38.6), (C-S). The number of small nodules (< 1 mm) was highest in LI-S, with 60.4 (31.8) nodules. LI-F and C of both varieties had between 34.4 and 40.6 small nodules.

Shoot length decreased significantly by 23.3% in EI-F and by 46.4% in EI-S, in relation to the respective control plants (Table 6). Number of pods decreased significantly in EI-F by 56.8% and in EI-S by 87.7%. LI-S differed significantly from both EI, but not from controls. Yield per plant was lowest in EI-S with significance toward LI-S and both controls and had a decrease of 96.6%. EI-F and LI-F differed significantly from both controls and decreased by 72.7% and 77.6%, respectively. LI-S decreased significantly by 58.9%. TKWs

Table 6 Effect of early (EI, BBCH 13) and late (LI, BBCH 62) PNYDV-infections and its increase or decrease compared to control on plant and yield parameters in *V. faba* variety ‘Fuego’ (F) and ‘GL Sunrise’ (S) in a small-scale field experiment

	‘Fuego’			‘GL Sunrise’		
	EI	LI	C	EI	LI	C
Shoot length (cm)	69.9 (5.63) b*	74.1 (6.54) abc	91.1 (2.78) a	45.4 (6.55) c	82.3 (3.16) ab	84.7 (1.96) ab
in-/decrease (%)	↓ 23.3	↓ 18.7		↓ 46.4	↓ 2.8	
Pods per plant	10.9 (2.41) c	15.3 (3.37) bc	25.3 (3.35) ab	4.5 (1.68) c	29.4 (5.12) ab	36.6 (3.98) a
in-/decrease (%)	↓ 56.8	↓ 39.4		↓ 87.7	↓ 19.6	
Yield of full kernels per plant (g)	6.71 (2.84) cd	5.51 (1.75) cd	24.59 (4.01) ab	1.13 (0.55) d	13.54 (3.38) bc	32.98 (4.51) a
in-/decrease (%)	↓ 72.7	↓ 77.6		↓ 96.6	↓ 58.9	
TKW of full kernels (g)	278 (29.3) bc	234 (21.3) c	398 (24.4) a	218 (14.6) c	269 (17.5) c	378 (18.2) ab
in-/decrease (%)	↓ 32.2	↓ 41.2		↓ 42.3	↓ 28.8	
N content of full kernels (% DM)	4.58 (0.23) bc	4.25 (0.19) c	5.03 (0.13) ab	4.06 (0.21) c	4.31 (0.10) c	5.27 (0.14) a
in-/decrease (%)	↓ 8.9	↓ 15.5		↓ 23.0	↓ 18.2	
quantity share of shriveled kernels (%)	36.8 (9.28) abc	55.2 (6.74) a	23.5 (3.36) bc	37.5 (7.69) ab	39.7 (2.69) a	16.5 (2.01) c
in-/decrease (%)	↑ 56.6	↑ 134.9		↑ 127.3	↑ 140.6	

Adjusted mean and standard error in brackets calculated with emmeans

EI early infection, LI late infection, C control

*Values with same letters in the same row are not significantly different at 5% level using emmeans

↑: increase

↓: decrease

of full kernels of control plants were significantly higher than those of infected ones and did not significantly differ over the varieties. EI-F decreased by 32.2% and LI-F by 41.2%. In ‘GL Sunrise’, TKW decreased by 42.3% in EI-S and by 28.8% in LI-S. Nitrogen concentration in full kernels was highest in the controls. In ‘Fuego’, these differences

were not significant. EI-S decreased by 23.0% and LI-S by 18.2%, both significantly. Quantity share of shriveled kernels was highest in LI with significant increases of 134.9% in ‘Fuego’ and 140.6% in ‘GL Sunrise’. EI was between LI and C and increased non-significantly by 56.6% in EI-F and significantly by 127.3% in EI-S.

Fig. 4 Plant samples of core (left), perimeter (middle) and reference (right) of five natural PNYDV foci in the on-farm investigation, 2018

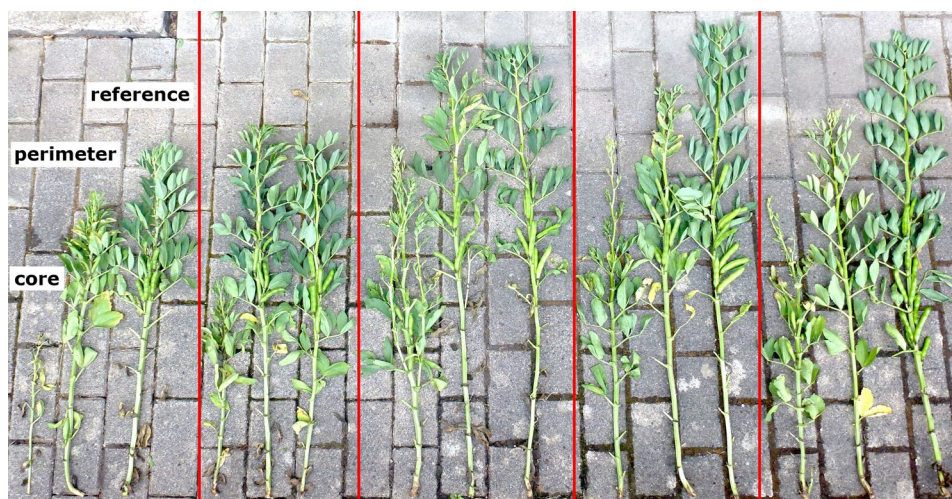


Table 7 Virus incidence (%) in PNYDV-infected plants and non-infected control plants of two *V. faba* varieties ‘Fuego’ and ‘GL Sunrise’ in a small-scale field experiment, 2020

	‘Fuego’		‘GL Sunrise’	
	PNYDV-infected	Control	PNYDV-infected	Control
PNYDV	100	0.0	100	0.0
PEMV	25.0	20.0	5.0	15.0
TuYV	0.0	5.0	0.0	0.0
BLRV	0.0	0.0	0.0	0.0

PNYDV pea necrotic yellow dwarf virus, *PEMV* pea enation mosaic virus, *TuYV* turnip yellow virus, *BLRV* bean leafroll virus

Virus detection

V. faba plants from naturally occurred PNYDV-foci showed severe symptoms in the core of stunting and yellow chlorosis (Fig. 4). The lower leaves appeared normal in size, but in the upper half of the stem, leaves were severely stunted, curly, narrow and stiff upward. Plants from perimeter had the same symptoms, but less pronounced. Here, only the upper tip of the shoot was stunted. The reference plants did not show any obvious virus symptoms.

In the small-scale field experiment, the infection of the plants was confirmed. Using ELISA, PNYDV was detected in 100% of the inoculated plants and 0% of the control (Table 7). Naturally occurring PEMV was more prevalent in the variety ‘Fuego’ (20–25%) than in ‘GL Sunrise’ (5–15%). TuYV was scarce, while BLRV was not found.

In two growing seasons, 2018 and 2019, naturally occurring PNYDV-foci were analyzed in an on-farm investigation. Three plants each from core, perimeter and reference of single foci were examined as a pooled sample by ELISA. In both seasons, core and perimeter were affected by PNYDV to 100% (Table 8). In the reference, PNYDV was also present to a high degree: 80% in 2018 and 100% in 2019. PEMV was detectable in 20–80% of the samples in 2018 with the highest value in the reference and to 50–75% in 2019 with highest values in core and reference. Over the two seasons,

TuYV was only found in the reference in 2018 and BLRV in the perimeter in 2019.

Discussion

Nodule number and weight

PNYDV had a consistent and significant negative effect on number and weight of faba bean root nodules in both varieties tested. The effect was demonstrated in three independent experiments under greenhouse, field and on-farm conditions. Such effects are also known for other viruses infecting faba bean (Abd El-Ghaffar et al. 2011; Ahmed 1986; Elsheikh and Osman 2002; Gomaa et al. 2006; Ismail and Atef 1998) and on other legumes (Gibson 1981; Guy et al. 1980; López et al. 2017; Mayoral et al. 1989; Taiwo et al. 2014; Tu and Ford 1984). Within all three experiments, in early infected plants, harvested at BBCH 70-76, the number and weight of nodules decreased by almost half to more than 90%. Nodules of late infected plants were less affected: nodule number partially increased and had the highest decrease by 17.8%. Nodule weight decreased by 18.5–34.4%. The effects of the time of infection have also been reported for other viruses (Franz et al. 1997; Frowd and Bernier 1977; Tu 1970; Tu and Ford 1984). Naturally occurring PNYDV foci are attributable to single infections by alate aphids, introduced in the core and spread over time from plant to plant, resulting in the perimeter (Saucke et al. 2019). Therefore, the core can be considered as an earlier and the perimeter as a later infection time point. However, we do not know at which stage the plants were infected, so we cannot equate the core and perimeter fractions directly with the early (EI) and late (LI) inoculation of the field experiment. In the greenhouse experiment, plants that were assessed at t2, the beginning of pod development (BBCH 70-73), were more active in nodulation with higher number, weight and red pigmentation of nodules, than those assessed at t1, at onset of flowering (BBCH 60-63). Thus, the stage of the beginning of fruit development can be regarded as a most suitable stage for nodule assessments in faba bean. In the on-farm investigation, the reduction in

Table 8 Virus incidence (%) in *V. faba* ‘Fuego’ on-farm in two growing seasons (2018 and 2019) in core, perimeter and reference zone of natural infection foci

	2018			2019		
	Core	Perimeter	Reference	Core	Perimeter	Reference
PNYDV	100	100	80.0	100	100	100
PEMV	20.0	20.0	80.0	75.0	50.0	75.0
TuYV	0.0	0.0	20.0	0.0	0.0	0.0
BLRV	0.0	0.0	0.0	0.0	25.0	0.0

Each sample site consisting of three pooled plant samples

PNYDV pea necrotic yellow dwarf virus, *PEMV* pea enation mosaic virus, *TuYV* turnip yellow virus, *BLRV* bean leafroll virus

nodulation was higher in 2019 than in 2018, which could be caused by an earlier spring flight of aphids and thus an earlier infection of the plants in 2019. In addition, other factors such as different nitrogen availability in the soil may also be causative for the difference in nodulation.

Nodule size

Nodule size was calculated from the quotient of weight and number of nodules. It appears contradictory in the different experiments. While nodules of PNYDV-infected plants were considerable larger than the controls in the greenhouse, they were significantly smaller in the field. Tu and Ford (1984) described that different viruses affect nodule size in different ways. An increase in size could compensate for a decreased number of nodules. This indicates that the results from greenhouse trials cannot be applied directly to the field situation, since environmental conditions such as soil structure, and microorganisms, as well as illuminance and precipitation, etc., have an influence on the nodulation (Seehuber 2015).

Pigmentation

In all three experiments, after early PNYDV infection, the total number of nodules not only decreased, but in particular the number of red nodules declined. These results agree with other studies in which virus infections led to a reduced level of leghemoglobin (Abd El-Ghaffar et al. 2011; Tu and Ford 1984). Nodule pigmentation gives an indication of their functionality (Virtanan and Laine 1946). Healthy, active nodules are red pigmented by leghemoglobin, which is essential for N₂ fixation (Wittenberg et al. 1974). *V. faba* has indeterminate nodules with distal a persistent meristem and proximal an older senescent zone (Hirsch 1992). Getting senescent, leghemoglobin is degraded by heme oxygenase (Baudouin et al. 2004; Kikuchi et al. 2005) and nitration (Becana et al. 2015) to green derived pigments. Consequentially, green nodules have previously been red and contained leghemoglobin. While the presence of leghemoglobin does not necessarily indicate current N₂ fixation activity, it shows that within their natural life cycle, nodules are healthier and less senescent (Puppo et al. 2005). At t1 in the greenhouse experiment, many white nodules were observed that represented an earlier nodule stage. Their amount was highest in control plants, which is in line with the observations by Joshi et al. (1967) who showed that white nodule development was faster on uninfected nodules of white clover than infected with clover phyllody virus (CPV). In contrast to greenhouse conditions, the proportion of degrading nodules was high in the field experiment 2020 in both varieties and even dominated the fraction of healthy nodules in the

on-farm assessment in both years 2018 and 2019. In our visual inspections, several nodules were affected physically by larvae of the pea leaf weevil (*Sitona lineatus* L.) feeding on root nodules. These represent an additional biotic stressor and may cause increased new nodule formation (Lohaus and Vidal 2010). This could make the results vague, and interactions between the impact of virus and feeding damage on the nodules cannot be excluded. In the field experiment, the LI showed only a slight decrease in red nodules in contrast to the control. But in the on-farm investigation, the plants of perimeter had no red nodules, while the reference still had approximately 24 red nodules per plant. Green and degrading nodules were highly present in all areas, which indicates an overall more proceeded senescence than in the field experiment. However, the proportion of green and degrading nodules was considerably higher in the perimeter than in the core. From these observations, it can be concluded that late infected plants do indeed form red nodules, but that they decrease earlier. Thus, PNYDV negatively affects leghemoglobin appearance, the more the earlier the infection occurs.

N₂ fixation

$\delta^{15}\text{N}$ and %Ndfa (nitrogen derived from atmosphere) indicate the amount of symbiotically fixed N₂ in plant biomass. A comparatively high $\delta^{15}\text{N}$ value corresponds to a large discrepancy between the isotope ratio of the sample and the atmospheric ¹⁵N abundance. This indicates a low assimilation of atmospheric nitrogen and results in lower %Ndfa values (Shearer and Kohl 1986). %Ndfa was calculated with a *B* value of -0.08 , that had been determined for ‘Fuego’ (Büchi et al. 2015). However, *B* values are subject to wide variation, even within species (Nebiyu et al. 2014), therefore, a different *B* may be appropriate for ‘GL Sunrise’. Other factors, like growing conditions and rhizobial strain(s), may also influence *B* (Unkovich and Pate 2000). For *V. faba*, other research revealed much lower *B* values (Fan et al. 2006; López-Bellido et al. 2010), so any employment of an experimental *B* assessment (Büchi et al. 2015) for calculating %Ndfa in a field, environment has to be taken with reservation. On the other hand, for the presented relative comparisons within a given variety, such *B*-related artifacts pose a constant factor. Parallel to the reduction in nodulation and leghemoglobin content, an increase in $\delta^{15}\text{N}$ and decrease in %Ndfa is expected. In the field and the on-farm investigation, this effect was manifested in a gradual increase of $\delta^{15}\text{N}$ from C/reference to LI/perimeter to EI/core plants, although the differences were not always significant; vice versa, %Ndfa and nitrogen concentration in shoots decreased. We can summarize that PNYDV, particularly an early infection, has a negative effect on nodulation and thus on N₂ fixation. This leads to a lower nitrogen concentration in shoots, although the plants have shorter shoots and produced less biomass.

Yield

In the greenhouse experiment, the yield decreased strongly by PNYDV to almost zero. Here, even the enlarged nodules could not compensate. In the small-scale field experiment, the number of pods per plant and the parameters of full kernels, yield, TKW and nitrogen concentration decreased in all infected variants and lowest in EI-S. Number and mass of nodules are correlated to plant performance (Allito et al. 2021). Comparing nodule weight and grain yield of the respective cultivars, the decrease in early inoculated plants is similar, approximately 72–82% for EI-F and over 93–96% for EI-S. However, this situation is different for late inoculated plants. Here, the decrease in nodule weight is only 34.4% (LI-F) and 26.9% (LI-S), while the decrease in grain yield is much higher, 77.6% (LI-F) and 58.9% (LI-S), respectively. This means that late infected plants at BBCH 62 still had a high nodule mass with a similar red pigmentation as the controls, but this did not prevent the final yield decline. Shriveled kernels are much smaller than normal filled kernels, whereby they will not contribute to yield when harvested with a combine during threshing. Late infected plants had the highest quantity of shriveled kernels, more than double than the control. We assume that these plants still created several grains, but they lacked the resources to fill them. Our results are comparable to Saucke et al. (2019), who performed an on-farm investigation with natural occurring PNYDV foci in 2016. They obtained yield decreases of 80 and 87% in the core, which is about the average in our EI, and of 46 and 62% in the perimeter, which is less than we observed in LI. Their decrease in crude protein concentration was higher in the core (21 and 32%) and similar in the perimeter (15 and 18%). Altogether, PNYDV leads to a significant yield loss, although not as severe as obtained losses caused by the related *Faba bean necrotic yellows virus* (FBNYV) on faba bean, which amounted to 100% in a field experiment in Syria at a comparable inoculation time (Franz et al. 1997).

Comparison of varieties

Varieties with reduced PNYDV susceptibility would be an important countermeasure against this new disease in grain legumes. The variety ‘GL Sunrise’ has been included in this study because of empirical evidence for reduced symptom development and less yield impact in years with high PNYDV incidence. However, EI-S had lowest values in all nodulation and yield parameters. LI-S had a slightly higher yield than ‘Fuego’, but nitrogen concentration in shoot and kernels, as well as nodule weight and red pigmentation was similar with LI-F. Thus, for ‘GL Sunrise’, we could assign the same general susceptibility toward PNYDV as for ‘Fuego’ with no indications for enhanced tolerance

concerning the data of the parameters investigated. Nevertheless, the empirical evidence remains and the better field performance of ‘GL Sunrise’ at high PNYDV pressure can also be related to the interaction with aphids as vectors. As *A. pisum* vectors both PNYDV and PEMV as major virus diseases in faba bean, the virus status of the small-scale field experiment provides a first hint, since natural PEMV incidence was lower in ‘GL Sunrise’ than in ‘Fuego’. Thus, further studies on vector phenology and host acceptance as well as variety comparison under natural infection conditions are subject for investigation.

Virus detection

For the attribution of the described symptoms to PNYDV infection, it is important to record the entire virus status of the plants to exclude other viruses that occur in mixed infections. In the on-farm investigation, naturally occurring PNYDV foci were observed and it was expected that there was a PNYDV incidence of 100% in core and perimeter and a lower incidence in the asymptomatic reference. Core and perimeter met this expectation. However, PNYDV was also present in 80% (2018) to 100% (2019) of the reference samples. This could be explained by the fact that the plants were infected late and have therefore not yet developed any symptoms. In addition, they may not have developed symptoms because virus concentration was limited due to slow plant growth. Furthermore, one sample consisted of three pooled plant samples and a positive result does not distinguish whether one, two or three plants were infected. Anyway, the reference cannot be equated with an uninfected control. With a vector occurrence in the field, completely clean controls are only possible by countermeasures such as insecticides or nets. However, these would again have an influence on the plants and would not correspond to real conditions. Therefore, the reference serves as an asymptomatic, PNYDV low level comparison. PEMV also had a high incidence of 20–80% in the pooled samples in the on-farm investigation, which is in line with a field survey in northern Hesse of 2016 (Saucke et al. 2019).

The virus status of infected plants in the small-scale field experiment 2020 confirmed 100% PNYDV and in controls 0%, respectively, whereas TuYV and BLRV are scarcely recorded or absent. Natural PEMV incidence, however, was not evenly observed in both varieties, with a comparatively low incidence of 5–15% in ‘GL Sunrise’ and a higher one between 20 to 25% in ‘Fuego’. These plants have been removed from the analyzed data set. The observed symptoms of stunting, yellowing and leaf curling (Fig. 4) are consistent with the current descriptions of PNYDV (Gaafar et al. 2016; Saucke et al. 2019; Ziebell 2017) and differ strongly from the symptoms induced by PEMV (mosaic, translucent spots, enations) (Tornos et al. 2008).

Impact of PEMV

In the greenhouse trial, PEMV infection led to a considerable decrease in nodule number and weight at t1, the onset of flowering. At t2, the beginning of fruit development, nodule dry weight was significantly lower, but in nodule number, the PEMV infected plants nearly caught up with the control. The fraction of red pigmented nodules decreased, but not severe. Assessment of nodulation in the field and on-farm trials were made at BBCH 69-76, thus comparable with t2 greenhouse data. In some of the on-farm samples also, PEMV was confirmed, so we have to take into consideration that there might be negative effects beside a PNYDV infection. Additionally, there could be synergistic effects, resulting in symptoms that are of increased severity than in an additive manner (Cockbain et al. 1983; Syller 2012; Zhou et al. 2017), which we cannot estimate for the combination of PNYDV and PEMV. In yield parameters, after all, there were no differences between PEMV infected and control plants in the greenhouse. Therefore, in this study, we estimate the effect of PEMV on yield to be negligible.

Conclusion

Our results demonstrate for the first time that PNYDV has a severe negative impact on nodulation parameters, symbiotic N₂ fixation and yield in *V. faba*, especially when plants were infected early. In contrast, as PEMV infection of *V. faba* in greenhouse trials had non-significant effects on nodulation and yield, we conclude that PNYDV appears as the main detrimental factor for N₂ fixation and yield performance in sole and mixed infections. Compared to the standard variety ‘Fuego’, ‘GL Sunrise’ showed similar susceptibility to PNYDV and decreased field performance when infection was artificially induced by temporarily caged vectors. However, a lower expression of PNYDV symptomatology was previously observed in ‘GL Sunrise’. This discrepancy could be attributed to a different interaction with PNYDV vectors during the natural initial and/or secondary spread of the virus in the vegetation. Further investigations are required for a better understanding of the complex PNYDV-vector interactions in faba bean and other grain legumes. This issue is important, as climate change could enhance aphid vector populations and thus promote the spread of viral diseases in Europe in the future.

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Availability of data and materials All relevant data are within the paper.

Code availability Not applicable.

Declarations

Conflict of interest There is no conflict of interests regarding the publication of this article.

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