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SHORT COMMUNICATION

First detection of viral pathogens of chickpea in Germany

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Abstract
Legumes play a crucial role in agriculture and serve as a valuable source of protein for human and animal consumption. However, legumes are susceptible to various viral infections, which can cause significant losses in yield and quality. Chickpea (*Cicer arietinum* L.) is an important food legume worldwide. While its cultivation in Germany is still in its infancy, it is expected to increase due to its numerous advantages. It is adapted to drought conditions, is an attractive additional option for crop rotations in organic farming and is gaining popularity for regionally produced food products. However, there is little knowledge about the presence and potential agronomic impact of viral pathogens in chickpea grown under German conditions. This study is the first that investigated the prevalence of legume-infecting viruses on chickpea crops grown in Germany. In 2022 and 2023, we collected sample material from symptomatic plants at three locations and analyzed it for the presence of circular (ss) DNA viruses, such as pea necrotic yellow dwarf virus (PNYDV), using a combination of rolling circle amplification (RCA) and downstream restriction fragment length pattern (RFLP) analysis. Furthermore, we conducted a reverse transcription PCR assay to detect different legume-infecting RNA viruses. In 2022 PNYDV was detected in 5 plants, all from the 'Amorgos' chickpea variety, located near a pea field. RNA viruses were detected in 27 plants, mostly TuYV or PEMV-1. Double and triple infections were common, with one plant exhibiting a triple infection of PNYDV, TuYV and PEMV-1. Symptoms of viral infection included leaf yellowing, dwarfism and chlorotic spots, with coinfections leading to more severe symptoms. In 2023, pea, cowpea and chickpea samples were collected from Schleibnitz/Wanzleben, Saxony-Anhalt, along with various chickpea accessions and cultivars from ZALF experimental fields, where the presence of PSbMV, PEMV, PNYDV, BYMV and TuYV was confirmed across these hosts. These findings indicate that viral pathogens pose a significant threat to chickpea production in Germany, necessitating the development of resistant cultivars and integrated management strategies.

Keywords Plant virus · Chickpea · PNYDV · Nanovirus

Introduction

Chickpea cultivation in Germany is on the rise due to its nitrogen-fixing capabilities, protein content and high suitability for food products. The yield potential for chickpea in Germany was found to be 1.7–1.9 t ha^{-1} in a network of on-station trials across Germany, Switzerland and Austria in 2021 and 2022, with large variability (Reckling et al. 2024). Despite chickpea's yield potential and beneficial properties, its cultivation may face potential threats from various pathogens that can significantly impact the crop's quality and yield. Viruses detected in other legumes such as field pea might also be present in chickpea crops. Given the increased cultivation and significance of chickpeas, the exploration of these viral threats becomes even more critical. Globally, viruses causing 'stunt disease' such as chickpea chlorotic

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stunt virus (CpCSV) or leafhopper-transmitted viruses like chickpea chlorotic dwarf virus (CpCDV) are of major economic importance in chickpea production. These viruses often lead to severe symptoms including stunting, yellowing or reddening and poor pod set. While cucumber mosaic virus (CMV), bean yellow mosaic virus (BYMV) and pea seed-borne mosaic virus (PSbMV) are present worldwide, their impact on chickpea is generally minor. Additionally, nanoviruses have also been identified as substantial risks to chickpea production in regions such as West Asia, North Africa and parts of Europe (Chatzivassiliou, 2021; Cun 2022). In our study, we collected chickpea plant samples from three field sites in Germany and subjected them to a comprehensive testing procedure, including rolling circle amplification (RCA) combined with restriction fragment length pattern (RFLP) analysis, and reverse transcription PCR (RT-PCR). Our goal was to explore the presence and impact of viral pathogens, particularly PNYDV, in German chickpea crops, with the aim of contributing to the development of effective mitigation strategies in the future. Recent studies (Cun 2022; Makkouk 2020) have enhanced our understanding of viral diseases in chickpeas, especially those transmitted by aphids. Moreover, they have highlighted the growing importance of coinfections, where multiple viruses exacerbate disease severity, leading to greater yield losses. These studies have also expanded the geographic awareness of viral threats to chickpea cultivation and refined diagnostic tools, enabling more accurate and earlier detection of viral infections. This deeper understanding is crucial as chickpea cultivation expands into new regions under changing climatic conditions.

In field pea (*Pisum sativum*), recent findings in Germany highlight the prevalence of pea enation mosaic virus 1 (PEMV-1, Enamovirus, *Luteoviridae*) and 2 (PEMV-2, Umbravirus) as the primary viral threats, followed by pea necrotic yellow dwarf virus (nanovirus, *Nanoviridae*) and turnip yellows virus (TuYV, Polerovirus, *Luteoviridae*), which also extends to non-legume weeds in the vicinity (Gaafar et al. 2016, 2020; Grigoras et al. 2014, 2010). The detection of an emerging emaravirus (Gaafar et al., 2020) in symptomatic pea plants across multiple growing seasons in a specific region underscores the evolving nature of viral threats to legume crops. Importantly, the majority of these identified viruses are aphid-transmissible, which underscores the complexity of their management and control. To date, there has been no specific report of viral infections in chickpeas in Germany. Upon inquiry, farmers cultivating chickpeas reported noticeable symptoms in their plants. These responses motivated us to investigate the affected crops specifically for potential viral infections. Our primary focus was PNYDV, a nanovirus that can infect chickpeas under laboratory conditions (Grigoras et al. 2014). Given the virus' multipartite genome structure and increasing prevalence in Europe (Saucke et al. 2019, Gaafar et al., 2020), especially Germany, understanding its occurrence and impact on chickpeas is crucial.

Materials and methods

Sample collection

In 2022 and 2023, symptomatic and asymptomatic chickpea plant samples were collected from three locations in Germany, two belonging to an organic farm in Schleibnitz/Wanzleben, in the state of Saxony-Anhalt, and one from the research station of the Leibniz Centre for Agricultural Landscape Research (ZALF) in Müncheberg, in the state of Brandenburg, Germany, to investigate the presence of viral pathogens, specifically circular single-stranded (ss) DNA viruses, such as pea necrotic yellow dwarf virus (PNYDV) and various common legume-infecting RNA viruses.

Circular ssDNA virus detection

Total nucleic acid extracts (Krenz et al. 2014) were prepared from individual chickpea plants that were either symptomatic or asymptomatic. To detect circular ssDNA viruses, RCA was employed to amplify the circular viral genomes. The amplified products were then digested using *Bst*UI restriction enzyme (NEB) followed by restriction fragment length pattern (RFLP) analysis (Haible et al. 2006) to identify specific viral signatures. In addition, a PCR was performed with Q5® high-fidelity DNA polymerase (NEB) to specifically amplify the M-Rep, -M or -S ORFs (see also Suppl. Table S1 primers and PCR program).

RNA virus detection

RT-PCR was conducted to detect different legume-infecting RNA viruses. Diagnostic primers for potyviruses, luteo- and poleroviruses, tospoviruses and specific primers for PEMV-1 were used (Abraham et al. 2006; Chu et al. 2001; Ha et al. 2008). Total RNA was extracted from the plant samples using Qiagen RNeasy Plant Mini kit, following the manufacturer's instructions. The extracted RNA was then reverse-transcribed into cDNA using reverse transcriptase and random hexamer primers or polyT primer. The resulting cDNA was used as a template for PCR amplification with the respective diagnostic and specific primers (Suppl. Table S1). The PCR products were separated on a 1.5% agarose gel electrophoresis, and the presence of specific bands corresponding to the expected amplicon size for each virus group or PEMV-1 was considered indicative of viral infection. To confirm the identity of the detected viruses, Sanger sequencing (Microsynth Seqlab, Germany) was performed

on selected PCR products. Sequencing data obtained from Sanger sequencing were analyzed (Geneious Prime® 2023.1.1) and confirmed the identity of respective viruses.

Virus transmission and greenhouse experiments

Inoculation of PNYDV from infected field pea to 12-day-old chickpea plants was carried out with green pea aphids (*Acyrthosiphon pisum*). Each chickpea plant was exposed to ten viruliferous aphids that had been exposed to PNYDV-infected pea plants for three days. The chickpea plants were then sprayed with contact insecticide after three days of inoculation. Monitoring of plant growth was carried out until the samples were collected after three months, and virus infections were confirmed using RCA/RFLP and PCR tests, matching the results from the ELISA tests performed before the experiments.

ELISA

For the detection of PNYDV in chickpea plants under greenhouse condition, samples with 100 mg of the new emerged leaves were collected three weeks post inoculation. Double-sandwich ELISA was carried out using the PNYDV-specific polyclonal antibodies and conjugate (JKI-1604) following the protocol according to Casper & Meyer (1981).

Results

From the 62 chickpea plants collected in 2022, we analyzed 48 and discovered that 28 were infected with one or more viruses. These infections ranged from single to triple infection across samples. PNYDV, a nanovirus, was detected in 5 out of the 48 plants, all of which belonged to the 'Amorgos' chickpea variety and were located near a pea field. The initial detection was made using RCA/RFLP analysis and further confirmed with PCR tests that targeted three distinct genomic segments of the virus: the ORFs of either DNA-R, DNA-M or DNA-S (Figs. 1, 2 a–d). The PCR results confirmed the detection, showing expected amplification of the aforementioned segments. Sequencing of the amplified PCR products and comparison with reference PNYDV sequences from GenBank further validated the presence of PNYDV. By RT-PCR screening, we identified RNA viruses in 27 out of the 48 chickpea plant samples (Figs. 1, 2 e–h). The detected viruses were: TuYV, found in 23 samples; PEMV-1, found in 19 samples; and ClYVV and BYMV each found in one sample. As expected, no tospoviruses were detected in any of the samples. Specific infection patterns were as follows: One sample had a single PNYDV infection, one sample displayed a PNYDV and BYMV double infection, two samples had double infections of PNYDV and TuYV, and one sample showed a triple infection of PNYDV, TuYV

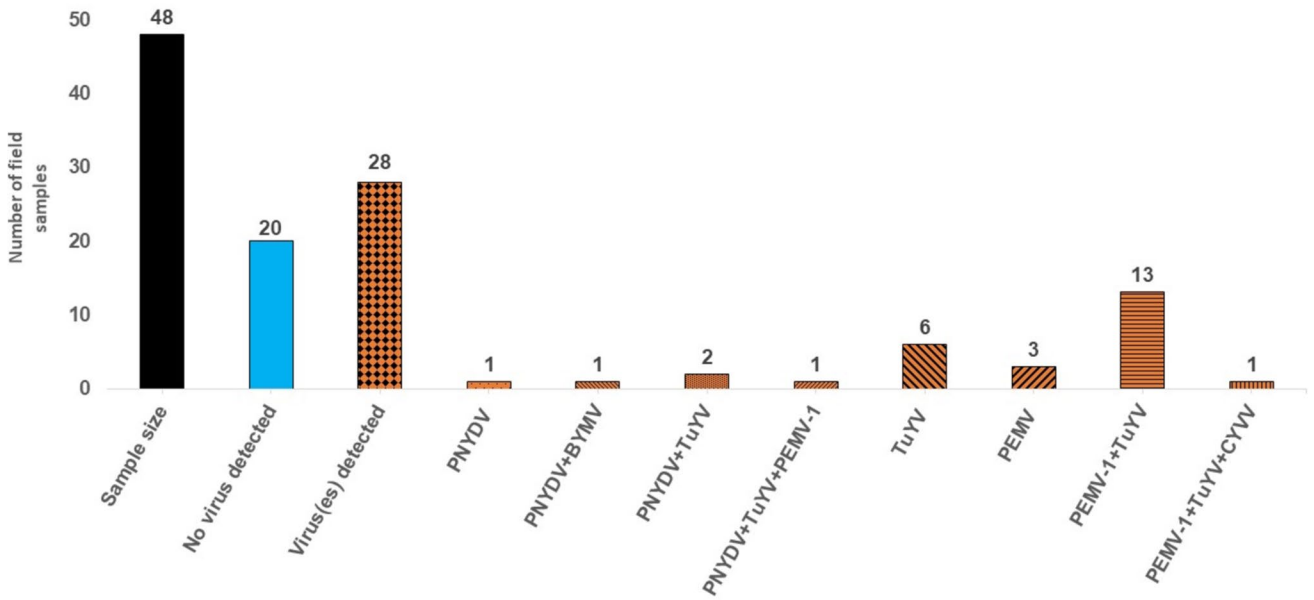

Fig. 1 Bar chart illustrating the prevalence of viral infections in the tested chickpea samples (n = 48) collected in 2022. The chart reveals that out of the 48 samples, 20 were negative for viral infections, whereas for the remaining 28 samples various viral infections could be confirmed. The detailed breakdown is as follows: one single infection with PNYDV, one double infection of PNYDV and BYMV, three double infections of PNYDV and TuYV, one triple infection with PNYDV, TuYV and PEMV-1, six single infections with TuYV, three single infections with PEMV-1, 13 double infections of PEMV-1 and TuYV, and one triple infection with PEMV-1, TuYV and ClYVV. The chart provides a visual representation of the varying degrees and combinations of viral infections found in the chickpea samples

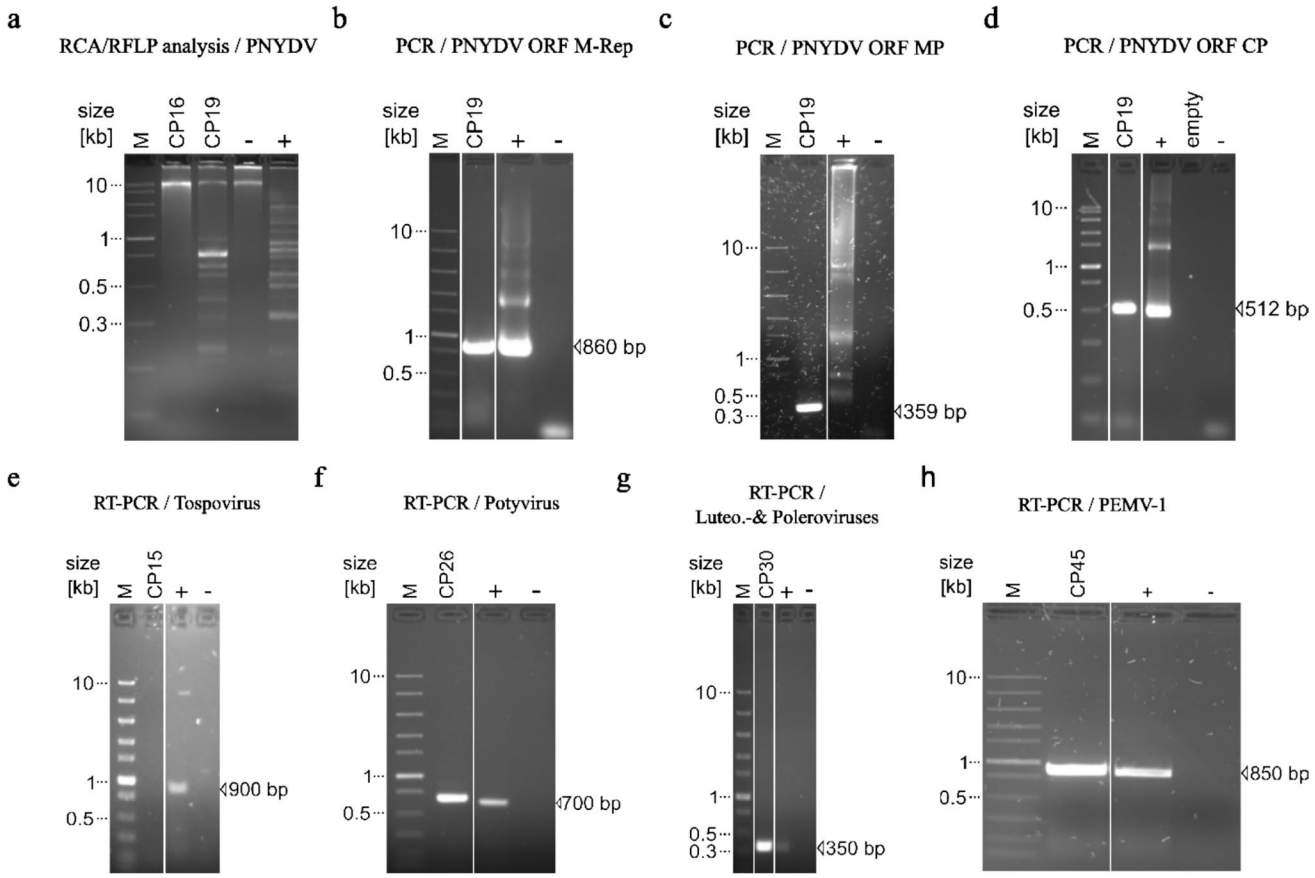

Fig. 2 Agarose gel electrophoresis images demonstrating the detection of pea necrotic yellow dwarf virus (PNYDV) and various RNA viruses in chickpea samples. Each image is representative or has been appropriately adjusted for clarity; full-scale images with all samples are available in the Supplementary Materials. **a** Rolling circle amplification followed by restriction fragment length pattern (RCA/RFLP) analysis using *Bst*UI restriction enzyme, indicating PNYDV infection. **b–d** PCR amplification of distinct PNYDV genomic segments: **b** DNA-R, **c** DNA-M and **d** DNA-S. **E–H** Detection of RNA viruses using reverse transcription polymerase chain reaction (RT-PCR): **e** Tospoviruses, **f** pea enation mosaic virus 1 (PEMV-1), **g** potyviruses and **h** luteoviruses. In each image, '+' denotes the positive control, while '-' represents the negative control (water). The presence of bands in the '+' lanes and the absence in the '-' lanes validate the specificity and efficiency of the respective PCR tests in amplifying target genomic segments

and PEMV-1. Six samples were solely infected with TuYV, three with only PEMV-1, 13 showed double infections of PEMV-1 and TuYV, and one sample presented a triple infection of PEMV-1, TuYV and ClYVV.

While healthy chickpea plants can be characterized by their average height of approximately 60 cm and a rich, vibrant green color, we found a clear impact of viral infections with chickpeas exhibiting varying symptoms (summarized in Suppl. Fig. 3). Chickpea plants infected with PNYDV exhibited notable yellowing of the leaves, a typical symptom of this virus. In addition, these plants displayed symptoms of dwarfism, further suggesting the presence of PNYDV. TuYV also caused observable distress in the infected chickpea plants. Although the symptoms were somewhat similar to those caused by PNYDV, plants infected with TuYV had a slightly paler appearance rather than the distinct yellowing associated with PNYDV. When chickpea plants were coinfected with both PNYDV and TuYV, the symptoms were even more severe, suggesting an additive or synergistic effect of the two viruses. Finally, we observed the effects of the PEMV-1 on chickpea plants. The symptoms in PEMV-1-infected plants were similar to those previously described, but with one noticeable difference: the presence of chlorotic spots. When a plant was coinfected with PEMV-1 and TuYV, the symptoms were drastically severe, to the point that the plant appeared to be on the verge of death.

In a subsequent investigation (summarized in Suppl. Table 2, sample names 108–118) carried out in June 2023 again at the organic farm at Schleibnitz/Wanzleben, samples from various leguminous plants were processed and evaluated for the presence of viral pathogens. One field pea exhibited a diverse infection profile. The plant was found positive for potyvirus and PEMV infections.

Furthermore, RCA and PCR analyses revealed the presence of the nanovirus PNYDV. Sequence analysis confirmed the presence of PSbMV, PEMV and PNYDV. Two faba bean plants were positive for potyviruses and showed an identical profile for nanovirus infections, as indicated by positive RCA and PCR results for both the M-Rep and CP regions. Both samples were identified as having BYMV upon sequencing. The chickpea samples presented various results. Samples 112 and 115 were positive for polerovirus infection with BLRV being identified by sequencing. Sample 114 showed infections with a polerovirus and PEMV, with sequencing confirming the presence of TuYV and PEMV. Interestingly, sample 116 not only showed a polerovirus infection (identified as TuYV) but also tested positive for a nanovirus in both the M-Rep and CP genomic regions. Sample 117 displayed infections from both polerovirus (BLRV) and PEMV, whereas sample 118 had only PEMV infection. Sample 113, on the other hand, did not show any viral presence in the tested parameters.

In an assessment of samples provided by ZALF in 2023, 14 distinct samples were analyzed. These samples encompassed a range of accessions and cultivars, including chickpea accessions CIC 61, CIC 66, CIC 147, CIC 225, and varieties Nero, Orion and Irenka, as detailed in Suppl. Table S2. CIC 66 accession sample with symptoms was positive for the potyvirus infection, notably PsBMV, and was also tested positive for PEMV. In addition, the symptomatic CIC 61, CIC 147 and Nero plant samples were also positive for PEMV. Analyzing the samples Orion, with one asymptomatic (W HE) and three symptomatic plant samples (W UN1, W UN2 and screening) alongside Irenka, with one asymptomatic and one symptomatic plant variants, a uniform negative outcome was observed, insinuating an absence of the viruses under investigation. Summarizing the results from the ZALF dataset, a predominant part seemingly lacked PNYDV, polero- and potyviruses. Yet, specific samples exhibited recognizable PEMV presence and the symptomatic CIC 66 variant emerged as the sole PsBMV positive specimen.

To check the viability and transmissibility of PNYDV, infection studies were carried out under greenhouse conditions. Aphid transmission of PNYDV was executed on 49 chickpea plants, and samples were collected after a three-month period. Thirty plants were tested positive by ELISA, and 12 samples were additionally confirmed by both RCA/RFLP and PCR tests. In the greenhouse samples, PNYDV-infected plants generally demonstrated a reduction in height compared to uninoculated controls and developed yellowing leaves in varying degrees, in some instances downward leaf curling occurred.

Discussion

Across the two-year survey (2022–2023), a total of 72 plants were collected and analyzed from three distinct locations in Germany: two organic farm sites in Schleibnitz/Wanzleben, Saxony-Anhalt, and the research station at ZALF in Müncheberg, Brandenburg. Of the 72 plants, 43 tested positive for one or more viral infections, underscoring the high susceptibility of chickpeas to viral pathogens in German agricultural environment, a situation evidently caused by aphid-transmitted viruses. Our findings mark the first reported occurrence of PNYDV in chickpea crops in Germany. Not only the detection of PNYDV in 10 samples, particularly in the 'Amorgos' chickpea variety, but also the identification of other aphid-transmitted RNA viruses like TuYV and PEMV-1, which were present in the majority of the infected samples, underline the prevalence and severity of these infections in these crops, indicating a new threat to the evolving German chickpea cultivation. It was observed that one chickpea field was directly adjacent to a pea field, suggesting that the common aphid-transmitted viruses found in peas in Germany could also be transmitted to chickpeas. This phenomenon mirrors observations from other regions where proximity to various legumes has facilitated interspecies virus spread—a problem documented across the legume cultivation community (Chatzivassiliou, 2021). The need to address these virus infections is now apparent, and the current options for managing them are limited, underscoring the necessity for innovative solutions. Future strategies must be developed not only aimed at virus resistance, combating virus infections or their vectors but also at the broader spectrum of pests, such as *Helicoverpa armigera* (Patil et al. 2017). Screening for genetic material with no or low susceptibility and breeding is needed for developing chickpea varieties with a resistance to these viruses. Further research needs to disentangle the effect of genotype, environment and management and their interaction on the infection of viral pathogens (Das et al. 2019). It is anticipated that chickpea cultivation in Germany will intensify due to chickpeas' resilience to climatic stress factors such as heat and drought (Reckling et al. 2024)—traits that become increasingly important as global temperatures rise. However, this resilience also presents a paradox, as increased chickpea cultivation could inadvertently raise the risk of viral epidemics unless resistance is found. In summary, our study serves as an initiative for more targeted research and monitoring efforts in this area. By advancing our understanding of these viral pathogens and their impacts on chickpea production in Germany, we can contribute to the broader goal of securing sustainable legume cultivation in the face of changing environmental and

climatic conditions. Our findings emphasize the importance of supporting such efforts with adequate funding for diagnostics, research and outreach activities.

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