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Viral pathogen production in a wild grass host driven by host growth and soil nitrogen

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Summary

- Nutrient limitation is a basic ecological constraint that has received little attention in studies on virus production and disease dynamics. Nutrient availability could directly limit the production of viral nucleic acids and proteins, or alternatively limit host growth and thus indirectly limit metabolic pathways necessary for viral replication.
- In order to compare direct and indirect effects of nutrient limitation on virus production within hosts, we manipulated soil nitrogen (N) and phosphorus (P) availability in a glasshouse for the wild grass host *Bromus hordeaceus* and the viral pathogen *Barley yellow dwarf virus-PAV*.
- We found that soil N additions increased viral concentrations within host tissues, and the effect was mediated by host growth. Specifically, in statistical models evaluating the roles of host biomass production, leaf N and leaf P, viral production depended most strongly on host biomass, rather than the concentration of either nutrient. Furthermore, at low soil N, larger plants supported greater viral concentrations than smaller ones, whereas at high N, smaller plants supported greater viral concentrations.
- Our results suggest that enhanced viral productivity under N enrichment is an indirect consequence of nutrient stimulation to host growth rate. Heightened pathogen production in plants has important implications for a world facing increasing rates of nutrient deposition.

Introduction

Pathogen–host interactions are ubiquitous in nature (Daszak *et al.*, 2000; Anderson *et al.*, 2004; Weiss & Stoye, 2013) and can be modulated by nutrient availability (Smith, 2002; Hall *et al.*, 2009b; Pradeep Ram & Sime-Ngando, 2010; Refardt, 2011). Pathogen presence can cause dramatic alterations in host performance, including decreases in biomass production (Morris *et al.*, 2007), physiology and transport (Prats *et al.*, 2006; Parke *et al.*, 2007), and even reduced survivorship of future generations (Johnson *et al.*, 2007). However, specific characteristics of the host, such as identity, defense strategy and phenotype, can reduce microbial pathogen performance as well (Kelley & Shykoff, 1994; Gómez *et al.*, 2009; Cronin *et al.*, 2010; Mordecai, 2011). A significant component of the system is overlooked when these reciprocal effects between the pathogen and host are considered outside of their abiotic context (Johnson *et al.*, 2007; Burdon & Thrall, 2014). For example, nutrient availability can lead to increased pathogen load within hosts (Mitchell *et al.*, 2003) or alter the virulence effects of the pathogen on host productivity and survival (Frost *et al.*, 2008). Despite the diversity of potential outcomes, surprisingly few attempts have been made to quantify the direct limiting effect of host nutrient concentrations on pathogen replication (but see Clasen & Elser, 2007; McKenzie & Townsend, 2007). Our study explores the ecological significance of nutrient availability in a model wild grass–pathogen system.

Feedback loops within a pathogen–host–nutrient interaction are complex (Alexander, 2010). The total impact of changes in nutrient supply on an infected host may switch from positive to negative depending on the balance of direct growth benefits from nutrient additions and indirect fitness costs via the pathogen. If nutrient addition enhances pathogen replication, this may outweigh the potential benefit of nutrient addition on the host, as was the case for a downy mildew infection on a species of *Plantago* under high nitrogen (N) conditions (Mandal *et al.*, 2008). Alternatively, an abundant supply of nutrient resources could mitigate the negative impacts of a pathogen on its host. For example, grain yield was improved and the severity of a fungal root disease reduced for agricultural wheat grown under conditions of high N and phosphorus (P) availability (Brennan, 1992). The outcome of the interaction between host and pathogen may also be a function of the relative concentration of two limiting nutrients, if, for example, different nutrients are limiting for host and pathogen (Smith, 2007). In this case, an increase in the supply of one nutrient could increase pathogen replication, thereby increasing negative impacts on the host, whereas an

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increase in the supply of the other nutrient could increase host tolerance or resistance, thereby decreasing negative impacts on the host (Smith, 1993).

Nitrogen and P are often limiting nutrients for organismal performance because of their abundance in life's most basic molecules, and their relative scarcity in the environment. Recent work done at the global scale highlights the stimulatory effects of N and P on organismal performance across a wide variety of systems (Elser *et al.*, 2007). Pathogens may also respond to nutrient limitation either directly or, because they are internal residents of their host, indirectly. First, pathogens may experience direct nutrient limitation as a function of their hosts' internal stoichiometry (Clasen & Elser, 2007). Viruses are particularly rich in N and P (Nass *et al.*, 1998), as they are composed primarily of P-rich nucleic acids and N-rich proteins (Hurst & Lindquist, 2000), which underscores the potential for intra-host N and P limitation in virus construction. Alternatively, pathogens may be indirectly limited by the nutrient availability in the host's environment such that slower growing hosts support a smaller quantity of pathogens (Bratbak *et al.*, 1993; Wilson *et al.*, 1996; Hall *et al.*, 2009a).

Though traditionally understudied, recent advances in metagenomic analyses underscore the pervasive presence of viruses across host species ranging from terrestrial plants (Raybould *et al.*, 1999; Muthukumar *et al.*, 2009) and marine microorganisms (Kristensen *et al.*, 2010) to fungi (Al Rwahnih *et al.*, 2011) and humans (Lecuit & Eloit, 2013). Moreover, viruses represent an important class of emerging infectious diseases in plants, accounting for almost half of all newly identified pathogens (Anderson *et al.*, 2004; Rodoni, 2009). Nevertheless, relatively few studies of plant viruses have been conducted in natural systems (Malmstrom *et al.*, 2011; Alexander *et al.*, 2014). Instead, experiments have been conducted in agricultural croplands where soil fertilization tends to negate any potential nutrient limitation for the plants (Green *et al.*, 2005), largely restricting our understanding of pathogen–host–nutrient feedbacks to cases where nutrients are unlikely to be limiting for either pathogen or host. Furthermore, heavily-managed systems typically comprise a significantly lower species and genetic diversity relative to more natural host populations. Lowered genetic diversity is well known to be coupled with reduced variation for tolerance and resistance to enemies (Crutsinger *et al.*, 2006; Mitchell & Power, 2006), as well as for reduced phenotypic variation in nutrient use and growth (Zhu *et al.*, 2000). Thus, disease dynamics within these genotypically restricted systems are likely to function in fundamentally different ways when compared to more natural host communities and populations.

Previous work has indicated that many physiological processes vary with the phenotypic growth rate and size of the host (Wright *et al.*, 2004; Elser *et al.*, 2010). For example, plant nutrient concentration has been shown to be dependent on size (Coleman *et al.*, 1993) and reduced allocation by plants to nutrient-rich molecular machinery with increasing plant size is a classic phenomenon in the literature (Elser *et al.*, 2010). Host growth rate can also influence defense mechanisms against pathogenic infection (Borer *et al.*, 2014b; Cronin *et al.*, 2014). This suggests that host growth and nutrient availability may jointly influence host tissue chemistry and pathogen load within infected individuals.

In order to explore how nutrient resources directly alter virus response and host–virus interactions, we experimentally quantified the production response of the viral pathogen, *Barley yellow dwarf virus-PAV*, to varying nutrient conditions within a wild grass host. Specifically, we manipulated both soil N and P conditions across gradients expected to range from limiting to overabundance in order to study their singular and combined influence on the pathogen–host interaction. Increased nutrient supply to the host may impact obligate intracellular pathogens like viruses in one of two ways: either by directly increasing the supply of the nutrient available to the pathogen within host cells, or by altering other aspects of host physiology and growth. For this reason, we compared the relative importance of nutrient concentrations in the host tissue to nutrient concentrations applied to the soil on viral productivity. We predicted that viral productivity would be strongly limited by nutrient availability within the host environment due to the high concentration of N and P in viral particles (Hurst & Lindquist, 2000). Additionally, microbial pathogens are known to induce changes in carbohydrate and mass allocation (Sutton *et al.*, 2007; Chen *et al.*, 2010). Therefore, we anticipated a diminishing return on the benefits of soil nutrient concentration for host performance and allocation response as the pathogen load increased and the strength of the host–virus interaction grew. To this end, we explored the combined influences of nutrient availability and viral titer, as a metric of enemy pressure, on host biomass allocation to above- and belowground organs.

Materials and Methods

Study system

Barley and cereal yellow dwarf viruses (BYDV) are aphid-vectored, generalist viruses, from the RNA-virus family Luteoviridae, and are known to infect over 150 species of wild and agricultural grasses around the world (Irwin & Thresh, 1990; D'Arcy, 1995). The viruses induce a systemic infection in the host's phloem tissues after feeding by viruliferous aphids (Irwin & Thresh, 1990; Jensen & D'Arcy, 1995). BYDV is not vertically transmitted via seed (Gray & Gildow, 2003). Infection can decrease both plant height and root mass/length (Irwin & Thresh, 1990; Jensen & D'Arcy, 1995) in addition to disrupting sugar translocation within phloem tissue and weakening vascular bundle cells (Jensen & D'Arcy, 1995).

For this experiment, we used a single isolate of *Barley yellow dwarf virus–PAV*, hereafter referred to as BYDV for brevity, to infect experimental plants. The isolate was originally collected in the 1960s (Rochow *et al.*, 1971) and has since then been propagated *in planta*, undergoing approximately three transmission cycles per year in laboratory plants of *Avena sativa* L. cv 'Coast Black' (CBO, for coast black oats). It has previously been used in inoculation experiments (Chay *et al.*, 1996; Peiffer *et al.*, 1997; Rúa *et al.*, 2013). The virus isolate has not yet been sequenced and is not currently included in GenBank.

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Bromus hordeaceus L. (soft brome, soft chess) is an annual grass of Eurasian origin commonly occurring in California grasslands. It was chosen as the focal host for this study primarily due to its rapid maturation and ease of infection (Rúa *et al.*, 2013), therefore making it a tractable laboratory model species. Seed for this species was hand collected from several naturally occurring populations, including roadside, pasture and lake habitats at the Hopland Research and Extension Center in California during June 2010. It was subsequently stored at the University of North Carolina at Chapel Hill at 4°C before use. Our experiment used seed sourced from multiple naturally occurring maternal lines, in order to include as much representative genetic variation as possible from natural populations.

Nutrient treatments and soil preparation

This experiment was conducted in the glasshouse at the University of North Carolina at Chapel Hill. Individual plants were grown in D60 Deepots (Steuwe and Sons Inc., Tangent, OR, USA) filled with *c.* 900 ml of soil comprising a 50 : 50 mixture of steam sterilized sand and Sunshine Mix #1/LC-1 potting soil (Sun Gro Horticulture Canada Ltd, Agawam, MA, USA). In order to ensure that plant growth and virus production were strongly limited by N and/or P, potassium and micronutrients were added to bulk soil mixtures. Potassium sulfate (K_2SO_4) fertilizer (Winston Weaver Co., Inc., Winston-Salem, NC, USA) and Micromax micronutrients (The Scotts Company, Marysville, OH, USA) were added at rates of *c.* 0.5 g and 0.75 g per pot, respectively, to reflect naturally occurring values. Four seeds were initially sown into each pot; 10 d after sowing, they were weeded down to one individual per pot (Supporting Information Table S1). Individuals were chosen primarily to maintain similarly sized plants across the entire experiment, and secondarily to maintain individuals growing in the center of the pot.

Five levels ('High', 'Medium–High', 'Medium', 'Medium–Low', 'Low') of both N and P were factorially manipulated at the individual level to produce a total of 25 unique treatment categories (Table S2). The range of values used for the N gradient (Table 1) was chosen with the aim of reflecting the relatively high atmospheric deposition rates found world-wide (Borer *et al.*, 2014a). Because external inputs of P to most terrestrial ecosystems are limited, we assigned values for the P treatments by first pairing each level within the P gradient to its corresponding level within the N gradient (e.g. 'Low' P pair 'Low' N), and by then assigning a treatment value based on a standardized mass ratio of 2 : 1. This yielded 'Low' P treatment values that were half the 'Low' N treatment value, 'Low–Medium' P treatment values that were half of the 'Low–Medium' N, and so on for each level within the gradient. Factorial crossing of every level within the two gradients produced a matrix of applied nutrient ratios (Table S2). The lowest nutrient levels were purposefully chosen to represent the bare minimum necessary for treatment application. Each treatment was replicated twice within all five blocks to produce 10 replicates per treatment total. Blocks were rotated twice before harvest.

The amount of N was applied over a 6-wk period in order to avoid damaging the roots or crown of the plant through a single dosage of hyper-concentrated fertilizer. Ammonium nitrate (NH_4NO_3) was diluted into 5 ml of water for each pot, and applied once a week for 6 wk beginning 2 wk after the seed planting date (Table S1). P was applied as a single dose before planting (Table S1) as triple super phosphate ($Ca(H_2PO_4)_2$) (The Espoma Co., Millville, NJ, USA), which was crushed to a fine powder and spread several centimeters below the soil surface in each pot.

Table 1 Treatment levels used for each nutrient of this experiment

Nitrogen	N ($g\ m^{-2}\ yr^{-1}$)	NH_4NO_3 (g per pot yr^{-1})
Low	0.137	0.00150
Low–Medium	4.10	0.0450
Medium	8.19	0.0901
Medium–High	12.29	0.135
High	16.38	0.180
Phosphorus	**P ($g\ m^{-2}$)**	**$Ca(H_3PO_4)_2$ (g per pot)**
Low	0.068	0.002
Low–Medium	2.05	0.06
Medium	4.10	0.12
Medium–High	6.14	0.18
High	8.19	0.24

Left-hand column, elemental additions; right-hand column, compound form actually applied individually to experimental pots.

Inoculation

Viral inoculations were completed 3 wk after germination when each plant was at the 2–3-leaf stage (Table S1). BYDV transmission occurs solely through aphid feeding (Gray & Gildow, 2003). Initially nonviruliferous aphids of the species *Rhopalosiphum padi* were placed in covered dishes, where they fed on known, virus-infected plant tissue for 48 h. Five newly viruliferous adult aphids were then carefully transferred onto each experimental plant and caged with caps constructed of clear plastic and nylon mesh. Viral transfer was completed by allowing the aphids to feed on each experimental plant for 72 h, after which the plants were un-caged and immediately sprayed with a horticultural oil solution (SAF-T-SIDE, ClawEl Specialty Products, Pleasant Plains, IL, USA) to kill the aphids. Two CBO individuals per block were inoculated concurrently as procedural controls. One CBO underwent the same treatment with infected aphids as the experimental plants, whereas the second was mock-inoculated with aphids fed solely on uninfected plant tissue.

Relative titer determination

In order to test the plants for successful BYDV infection and to quantify relative viral titers (concentrations of virus particles), a compound indirect double-antibody sandwich Enzyme-linked Immunosorbent Assay (ELISA) was performed using antibodies purchased from Agdia Inc., Elkhart, IN, USA, on 0.05–0.30 g aboveground fresh tissue from each experimental plant, as detailed in (Cronin *et al.*, 2010). Samples were homogenized through grinding in extraction buffer at a constant tissue:buffer

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mass ratio of 1 : 10. Due to procedural constraints, each block was assayed separately. Of the 250 *B. hordeaceus* plants that were inoculated with infected aphids, 58 did not become infected with BYDV and were consequently removed from the analysis, leaving 192 experimental plants. Diagnostic statistical models showed that there was no effect ($P > 0.10$) of either the N or P treatment on inoculation success or failure, nor was there any effect of the N and P treatments on aphid reproduction during the 3-d inoculation period. Among the procedural controls, all CBO plants that were inoculated with infected aphids were successfully infected with BYDV, whereas all CBO plants that were mock-inoculated with uninfected aphids remained uninfected.

Standard curves were used to estimate the titer of each infected experimental plant, relative to heavily infected plants of the agricultural host CBO. We used CBO for the standards because it is able to sustain much higher titers of BYDV (Cronin *et al.*, 2010; Rúa *et al.*, 2013). The standards were prepared as follows. Following the same ELISA procedure as for the experimental plants, fresh aboveground tissue from infected CBO plants was homogenized through grinding in extraction buffer, thereby extracting viral particles into the buffer. This initial extract represented a maximal relative titer value of 1.0, and was then diluted with healthy tissue extract from the same species (CBO) to yield the standards. These diluted standards varied in 1% increments from 1% to 20% infected tissue (corresponding to relative titers of 0.01–0.20). For example, the 1% standard was prepared using 1 part infected tissue extract and 99 parts healthy tissue extract, and so on. A standard with a relative titer of 0.50 was also prepared, in order to extend the range of possible relative titer values upwards from 0.20. These 21 standards were prepared separately for each block of experimental plants, and were run on the same ELISA plates as the experimental plants and procedural controls. Each ELISA plate included one set of 21 standards and all the experimental plants and procedural controls from one block of the experiment. The ELISAs yielded an optical density (OD) value for each sample that can be used as a metric of viral concentration (Copeland, 1998; Rúa *et al.*, 2013; Cronin *et al.*, 2014).

Different plant species can have differing values of background noise in their OD values. To facilitate comparison of the *B. hordeaceus* experimental plants to the CBO standards, we estimated and subtracted the background component of the OD values for each species, in order to compare only that component of the OD values which was caused by virus titer. For CBO, we calculated the average OD value for three healthy CBO, and then subtracted that average from the OD values of all 21 CBO standards. Experimental *B. hordeaceus* OD values were likewise adjusted using an average of three healthy *B. hordeaceus* OD values.

After subtracting the background component of the OD values, we fitted either a linear or quadratic regression model to each set of 21 standards, corresponding to a standard curve for each experimental block. Because we subtracted the background component of the OD values, each standard curve was forced through the origin. For Block 1, including the standard with a relative titer of '0.50' decreased curve fit and was not necessary to capture the range of OD values from the experimental plants. Therefore, relative titers for Block 1 were estimated using a standard curve without the '0.50' value. The standard curves allowed us to estimate virus titer, relative to the CBO standards, for each experimental plant (Fig. S1).

Plant harvest

All plant tissue was harvested 10 wk after planting (Table S1) and divided into shoot and root portions. Root portions were frozen at −20°C before being separated from their surrounding soil. Both above- and belowground masses were dried at 60°C in an oven for at least 72 h before weighing. All wet-weight plant tissue associated with the ELISA procedure was reincorporated into the final aboveground biomass using a dry-to-wet weight conversion factor. This conversion factor was calculated for each individual plant based on net aboveground mass lost during the drying procedure for shoot tissue not associated with the ELISA procedure.

In order to quantify the concentrations of N and P in the plants, the remaining shoot material was ground using a ball mill. Tissue N concentration was determined by subjecting 1.5–3.0 mg of material to Micro-Dumas Combustion, whereas tissue P concentration was determined using a colorimetric analyzer (Alpkem Corp., Clackamas, OR, USA) after dry ash/acid extraction (Stable Isotope/Soil Biology Laboratory of the University of Georgia's Odum School of Ecology). Due to logistical constraints, a subset of plants was selected for tissue chemistry analysis. Within each treatment combination of N and P addition, one plant was randomly selected from each of the five experimental blocks. Where no successfully infected plants were present for a single block, a replacement was randomly selected from another block, within the same treatment combination of N and P addition. To capture the full range of experimental variation, we used the extremes of the gradient for both soil N and P treatments ('Low', 'Low–Medium', 'Medium–High', 'High'). No plants were selected from the 'Medium' N- or 'Medium' P-treated plants, leaving 16 out of the original 25 treatment combinations of N and P addition. From each of the remaining 16 treatment combinations, five plants were selected, for a total of 80 plants. However, two plants with relative titer measurements above 0.40 were removed from tissue analysis because titer values this high could not be adequately estimated from our standard curves, leaving 78 experimental plants. Finally, one plant in the low soil-P treatment had the fourth highest tissue P concentration in the entire experiment, indicating contamination or error, so it was removed; this left 77 experimental plants ultimately included in our statistical analyses (Table S3).

Statistical analyses

Our modeling framework was chosen in order to investigate treatment-level effects of soil N and P addition on tissue %N and %P concentrations, viral titer and host root fraction. Host root fraction refers to the ratio of belowground mass to total plant biomass and is an indicator of plant allocation strategy. Relationships among variables were evaluated by fitting mixed-effects models with block as a random effect. All models were run using

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the 'lme' function from the 'nlme' package (Pinheiro *et al.*, 2014) in R v3.1.0 (R Foundation for Statistical Computing 2012, Vienna, Austria). The structure of all models and all model results are displayed in the Table S4(a–f). Host tissue N : P ratios were log-transformed in order to linearize the predictor variable. Likewise, total host biomass (i.e. the sum of root and shoot biomass, hereafter referred to as simply 'host biomass') was square-root transformed and relative viral titer was log-transformed in order to meet assumptions of normality and homoscedasticity. As a metric for goodness-of-fit for mixed models with one-level grouping, we report here R^2 values based on likelihood ratios ('lmmfit' package; Maj, 2013; Table S4). Soil N and P addition rates were treated as continuous variables, so each model fitted a surface in multidimensional space. Our core results center on two-way interactive effects involving N addition, which could each be visualized as a three-dimensional surface. However, such surfaces are difficult to visually interpret, so we took advantage of there being only four analyzed rates of N addition in order to project each surface into a two-dimensional plane, with each rate of N addition indicated by color.

Results

Host tissue nutrient concentrations

Soil N addition increased the concentration of N within host aboveground tissue (%N), but this effect greatly depended on host size and, to a lesser extent, soil P addition (Soil N × Host Biomass × Soil P : $F = 4.29$, $P = 0.0423$; $R^2 = 0.68$). Specifically, addition of soil P tended to increase %N at lower rates of N addition and for smaller plants, but decrease %N at higher rates of N addition and for larger plants. Interestingly, the negative association between host biomass and %N decreased with greater soil N availability; looked at another way, the positive impact of N addition on %N decreased with greater host biomass (Soil N × Host Biomass: $F_{1,65} = 9.67$, $P = 0.0028$; Fig. 1). Addition of soil P increased P concentration in aboveground tissue (%P) (Soil P: $F_{1,65} = 12.95$, $P = 0.0006$; $R^2 = 0.20$; Fig. S2). However, unlike %N, %P was not influenced by either soil N addition or host biomass (Soil N: $F_{1,65} = 0.619$, $P = 0.434$; Host biomass: $F_{1,65} = 1.68$, $P = 0.199$). We also assessed the role that soil nutrient addition played in altering host tissue N : P ratios. Increasing soil N led to higher host N : P (Soil N: $F_{1,65} = 12.4$, $P = 0.0008$) whereas increasing soil P availability lowered host N : P (Soil P: $F_{1,65} = 27.9$, $P < 0.0001$), as would be expected ($R^2 = 0.53$). Host biomass also had a stimulatory effect on host N : P ratios ($F_{1,65} = 35.7$, $P < 0.0001$), indicating that larger hosts were more N rich relative to P. Collectively these results indicate that the experimental treatments successfully altered internal host chemistry.

Viral titer

Experimental addition of soil N increased the titer of virus infections up to a threshold level. Across N treatments, viral titer also increased with host biomass. Moreover, the relationship between viral titer and host biomass depended on the level of N addition.

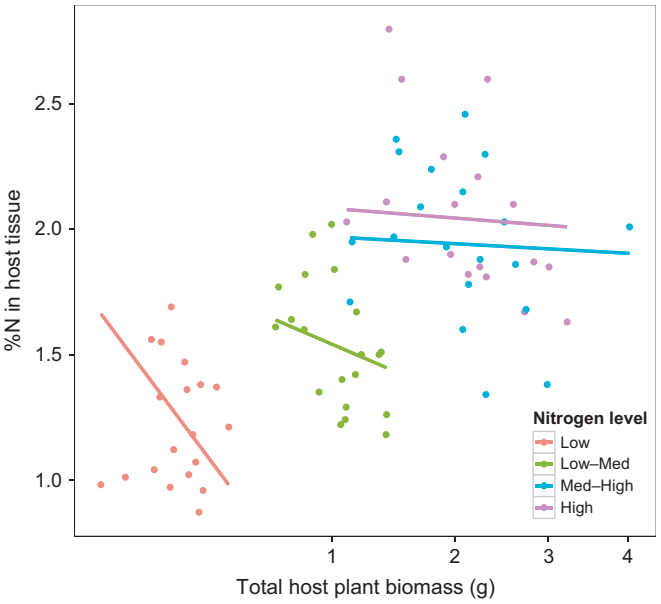


Fig. 1 Soil nitrogen (N) addition and host biomass increased aboveground tissue N content. Each point represents a single *Bromus hordeaceus* host individual. The biomass axis has been square-root transformed, but values shown are in untransformed units. Regression lines depict the relationship between host biomass and internal host N concentration (%N) for each soil-N treatment level.

At lower levels of soil N addition, viral titer was higher for larger hosts, whereas under higher levels of soil N addition, the pattern was reversed and viral titer was lower for larger hosts (Soil N × Host Biomass: $F_{1,65} = 5.32$, $P = 0.0242$; $R^2 = 0.23$; Fig. 2). By contrast, P addition had no significant effect on viral titer (Soil P: $F_{1,65} = 0.055$, $P = 0.815$).

In order to better understand the biological context of host–virus interactions *in planta*, a model linking aboveground host tissue chemistry to viral titer was explored as a complement to the experimentally manipulated soil nutrients model. Host biomass alone significantly influenced viral titer. Specifically, virus accumulation was greatest for larger hosts, independent of tissue N or P concentrations (Host Biomass: $F_{1,65} = 13.7$, $P = 0.0004$; $R^2 = 0.21$). After controlling for variation in host biomass, neither host %N or %P was correlated with viral titer (%N: $F_{1,65} = 0.970$, $P = 0.328$; %P: $F_{1,65} = 1.52$, $P = 0.222$).

Host root allocation

Increasing soil N decreased allocation to belowground plant biomass (Soil N: $F_{1,65} = 39.5$, $P < 0.0001$; $R^2 = 0.41$; Fig. S3). However, no effect of Soil P on root fraction was detected (Soil P: $F_{1,65} = 1.45$, $P = 0.236$). Viral productivity also influenced host biomass allocation, whereby relative investment in root mass decreased with increasing virus accumulation (Viral Titer: $F_{1,65} = 5.47$, $P = 0.0225$; Fig. 3).

Discussion

In our experiment, nutrient availability played an important role in altering the population productivity of a viral pathogen;

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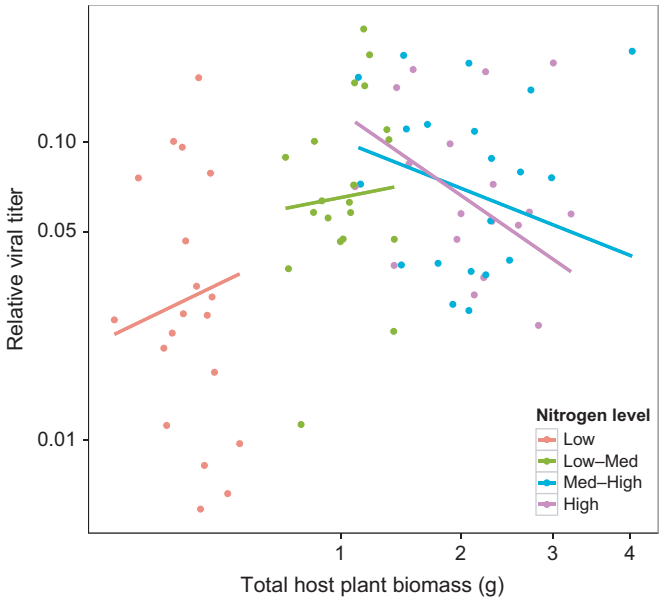


Fig. 2 Soil nitrogen (N) addition and host biomass increased viral titer. Each point represents a single *Bromus hordeaceus* host individual. The biomass axis has been square-root transformed and the relative viral titer axis has been log-transformed, but values shown are in untransformed units. Regression lines depict the relationship between host biomass and relative viral titer for each soil-N treatment level.

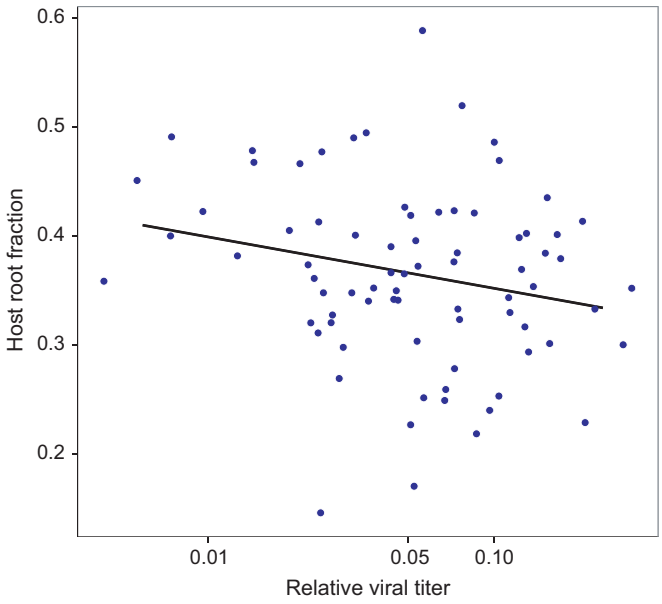


Fig. 3 Host root fraction decreased with increasing viral titer. Each point represents a single *Bromus hordeaceus* host individual. The viral titer axis has been log transformed, but values shown are in untransformed units. The regression line depicts the relationship between relative viral titer and host root fraction.

however, this role was largely mediated by host biomass production rather than nutrient concentration in host tissue. Virus production was augmented by N addition to the soil in association with greater host biomass, although ultimately we detected a diminishing return for viral productivity from both host biomass and soil N additions. In contrast to our predictions, virus titer was not correlated with host leaf tissue N or P concentration, but viral pathogen production was still strongly stimulated in hosts of greater size, presumably as a result of the faster growth rates of the larger hosts (Hall *et al.*, 2009b; Cronin *et al.*, 2010), and regardless of the concentration of N or P in the hosts. We did not find evidence for an interaction between the two resources in limiting viral production *in planta* (Smith, 1993), or in altering the strength of the host–pathogen interaction (Smith, 2007), as measured by changes to host root fraction. Nonetheless, host root fraction did decrease with increasing viral productivity. This result extends previous studies showing that BYDV infections typically reduce host allocation to root biomass, relative to uninfected host individuals (Jensen & D'Arcy, 1995; Rúa *et al.*, 2013; Cronin *et al.*, 2014).

We designed our experiment with the explicit purpose of creating a wide range of nutritionally variable virus habitats (i.e. hosts), in order to detect any population-level response of a viral pathogen within a grass host. This included incorporating N application rates well above those observed for many terrestrial systems in the post-industrial era (Bobbink *et al.*, 2010), although on a par with rates used in agricultural settings (Economic Research Service, 2013). Despite the wide range of controlled nutrient manipulations tested, we did not find any support for limitation of viral titer by either %N or %P in host leaf tissue. The lack of correlation between viral titer and leaf nutrient concentrations in our study may reflect the challenges of measuring nutrient supply rate to intracellular pathogens restricted to certain host tissues. Though BYDV is a systemic pathogen, it only replicates within the phloem tissue (Irwin & Thresh, 1990; Jensen & D'Arcy, 1995). Out of a technological necessity, our measurements for tissue N and P were pooled across multiple tissue types in the aboveground plant mass. Because tissue types differ in their relative nutrient composition (Hall & Baker, 1972; Lohaus *et al.*, 1995), it is possible that measurements of nutrient concentration within the phloem tissue alone may have revealed a direct effect of nutrient limitation on viral production. Future studies may consider the use of a modified aphid stylectomy technique (Turgeon & Wolf, 2009), whereby a phloem tap could be created and sap analyzed directly using nutrient assays and RT-PCR.

An alternative explanation for the lack of correlation between viral titer and leaf nutrient concentrations is that plant viruses are not actually limited by N and P concentrations directly, but rather by the availability of host cellular machinery, such as transporter and membrane traffic proteins (Krishnan *et al.*, 2008). Viruses are obligate intracellular pathogens that require the cellular machinery of their host to reproduce (Smith, 2007; Hao *et al.*, 2008; Cuomo *et al.*, 2012). Greater concentrations of photosynthetic and enzymatic machinery are present in faster growing organisms (Elser *et al.*, 2010), and increases in such cellular machinery are one mechanism by which plant growth rate increases with soil N addition (Yu *et al.*, 2012). In virus-infected plants, this positive effect of soil N addition on the availability of cellular machinery may also lead to greater internal virus loads.

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A direct role for host growth rate in driving the ecology of host–virus interactions is increasingly supported in the literature (Cable *et al.*, 2007). In a comparison study across amphibian species, the susceptibility to infection by a ranavirus increased with host development rate (Hoverman *et al.*, 2011). Similarly, quicker growing host individuals from among six host species supported higher BYDV loads via a reduced investment in pathogen resistance in a recent glasshouse study (Cronin *et al.*, 2014). Despite these results, the mechanisms driving increased viral prevalence across nutrient gradients in field experiments remain unclear. For example, increasing soil P availability was correlated with both a rise in BYDV infection prevalence and a shift in viral community diversity *in planta* (Borer *et al.*, 2010; Seabloom *et al.*, 2013). Another recent experiment showed that the prevalence of BYDV infection was also greater in soil fertilization treatments high in P relative to N (Borer *et al.*, 2014b). By contrast, our experiment found no effect of P on viral titer, indicating that the effects of P addition on viral prevalence in these field experiments are unlikely to have been caused by greater viral titer.

Our study specifically focused on the interaction of two key nutrients and host growth rate in mediating the ability of the pathogen to multiply within the host. To our knowledge, no other study has experimentally quantified the effects of two potentially limiting nutrients on viral pathogen abundance within terrestrial hosts. In one study of a freshwater algal host–virus system, host P stoichiometry (C : P) dictated viral productivity (Clasen & Elser, 2007). By contrast, N availability rather than P availability elicited a stronger viral production response in our terrestrial host–pathogen system. Our result showing little influence of P addition mirrors the result of a previous glasshouse experiment that tested the effect of soil P (but not N) addition on BYDV titer in *Bromus hordeaceus* and one other wild grass host (Rúa *et al.*, 2013). In another study manipulating single and double inoculations of BYDV-PAV and a related virus species in a grass host, success of co-inoculations was greatest for soil conditions high in N relative to P. Furthermore, manipulating soil nutrient conditions also provided evidence for ecological competition between viral isolates as a function of soil nutrient conditions (Lacroix *et al.*, 2014). In light of these results, our study elucidates one biotic mechanism by which virus performance is enhanced by high resource availability. (For a further review of published effects of nutrient addition on viral titer, see Table S5.)

Many epidemiological and ecological processes are influenced by environmental nutrient availability (McKenzie & Townsend, 2007), including not only pathogen productivity, but also susceptibility to infection (Hoffland *et al.*, 2000) and population cycling (Gerla *et al.*, 2013). As pathogens often infect multiple host species (Daszak *et al.*, 2000; Anderson *et al.*, 2004), the outcome of such nutrient effects may hinge on characteristics of those host species, such as evolutionary legacies of adaptation to nutrient conditions (Johnson *et al.*, 2010) and growth rate (Cronin *et al.*, 2014). The ecological limitations of the pathogens themselves should not be ignored either: virus species can also differ in their response to ecosystem-level changes in nutrient availability (Seabloom *et al.*, 2013). Across such potential diversity in functional response for host and virus species, our results suggest that variation in host growth rate, particularly in response to increased resource availability, may be an important consideration for future studies tracking host competence and disease transmission in natural communities (Gray & Gildow, 2003; Meentemeyer *et al.*, 2012; Cornet *et al.*, 2014).

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Standard curves relating raw optical density (OD) values obtained from an ELISA procedure to proportions of infected plant tissue (the standards).

Fig. S2 Relationship between aboveground tissue phosphorus content (%P) and soil phosphorus (Soil P).

Fig. S3 Relationship between host root fraction and soil nitrogen (Soil N).

Table S1 Experimental timeline

Table S2 Matrix of applied soil Nitrogen:Phosphorus (N:P) ratios obtained from crossing of the nutrient gradients

Table S3 *Bromus hordeaceus* plant numbers included in the final statistical analysis, arranged by soil nitrogen and phosphorus treatments

Table S4 Results for all statistical models assessed

Table S5 Short review of the published effects of nutrient addition on viral titer

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