Wheat’s wild relatives vary in their response to nitrogen and ozone

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Abstract

The wild relatives of bread wheat (*Triticum aestivum* L.) are valued by plant breeders for their genetic diversity. However, increasing levels of nitrogen (N) deposition and ground-level ozone (O_3) threaten plant biodiversity in the Mediterranean and Near East, a hotspot for many crop wild relatives. Knowledge of the effect of these air pollutants in combination is still limited, but early indications are that effects vary depending on the level of pollutants, and on the sensitivity of the species to N and O_3. This study examined the responses of four important wheat wild relatives (*Aegilops tauschii*, *Aegilops speltoides*, *Triticum dicoccoides*, and *Triticum monococcum)* and one modern wheat cultivar (*Triticum aestivum* ‘Cadenza’) to treatments of N (equivalent to 50 kg ha^{-1} yr^{-1} ammonium nitrate) and O_3 (100 ppb for 21 days), alone and in combination. Measurements included root, shoot and seed biomass, and electrolyte ratios. The O_3 sensitivity of *Ae. tauschii* and *T*. ‘Cadenza’ were exacerbated by the addition of N, whilst *Ae. speltoides* was found to be nitrophilous, with N ameliorating the negative effect of O_3. Both *T*. ‘Cadenza’ and *T. dicoccoides* produced immature seed heads, with the cultivar’s seed head biomass reduced in response to O_3 and N+O_3 whilst that of *T. dicoccoides* was largely unaffected. These data suggest that all four wild relatives are likely to be affected when N and O_3 air pollutants co-occur, and their *in situ* populations may therefore be at risk. Equally, the results of this study can inform use of their beneficial traits by wheat breeders, and alert them to the inadvertent inclusion of N and O_3 sensitivity.

Keywords

Crop wild relative; *Triticum aestivum L.*; *Triticum dicoccoides*; *Aegilops tauschii*; *Aegilops speltoides*; air pollution.

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Introduction

Wheat provides 20% of the global calorie and protein intake consumed by the current population of 7.5 billion people, with the majority supplied by just one species, *Triticum aestivum* L., bread wheat (Shiferaw et al., 2013). Whilst the green revolution delivered higher yields it also led to a substantial reduction in the genetic diversity found in wheat cultivars, making them more vulnerable to pest and disease attack and less adaptable to abiotic stress (The Royal Society, 2009). Wild populations of *Triticum* and *Aegilops* species which form the close wild relatives of wheat, have retained their wide genetic diversity and, as a result, are particularly useful to plant breeders tasked with both increasing wheat yields and broadening the narrow genetic profiles of wheat cultivars to improve resilience (Brozynska et al., 2015, Dempewolf et al., 2017). These species continue to grow in the eastern Mediterranean and Near East, a region containing a high proportion of important crop wild relatives (Castañeda-Álvarez et al., 2016). However, this region is also a convergence zone for global air pollutants, especially reactive nitrogen (N) and ozone (O_3) (Lelieveld et al., 2002), both of which are known to affect plant biodiversity (Stevens et al., 2004).

Many of wheat's close wild relatives have been extensively used for wheat improvement, mostly to improve pest and disease resistance (Maxted and Kell, 2009). Whilst the *ex situ* collections of wheat germplasm conserved in gene-banks around the world are considered to be well stocked (Castañeda-Álvarez et al., 2016), the importance of maintaining the wild populations *in situ* is now recognised as a high priority, both because the genetic diversity of the *ex situ* accessions is comparatively limited (Sasanuma et al., 2002) but also because, unlike the stored seed, wild populations continue to evolve and adapt to global change (Maxted et al., 2010, Castañeda-Álvarez et al., 2016). However, crop wild relative populations in the Mediterranean are under threat, with agricultural intensification, over-grazing, habitat loss, and climate change all contributing to a loss of genetic diversity (Jarvis et al., 2008, Stolton et al., 2008, Haruntyunyan et al., 2010, Redden et al., 2015). The additional threat from air pollution may be equally important, and affect species even in protected areas.

Over the past 100 years the burning of fossil fuels and increasing use of artificial fertilisers for agriculture have led to a doubling in the total fixation of N globally and a tripling of levels within Europe (Sutton and van Grinsven, 2011). With ongoing industrialisation and agricultural intensification in many parts of the world, levels of reactive N are expected to continue to increase (Sutton and Bleeker, 2013), with predictions that N deposition across the Mediterranean region, currently in the range of 12 – 45 kg N ha^{-1} yr^{-1}, could triple by 2050 (Galloway et al., 2004, Ochoa-Hueso and Stevens, 2015). Anthropogenic emission sources also contribute precursor gases, nitrogen oxides, methane, carbon monoxide and non-methane volatile organic compounds, which react with sunlight to form $O_{3.}$ Global O_3 daytime growing-season averages reach 30-50 ppb in the northern hemisphere (Sutton and van Grinsven, 2011) with occasional short term peak O_3 episodes of 100-200 ppb (The Royal Society, 2008). Some of the highest background levels of O_3 occur in the Mediterranean and Middle East, especially in summer when air streams from Europe, Asia and Africa converge on the region (Zanis et al., 2014). O_3 levels of 60-100 ppb are expected to occur in parts of the eastern Mediterranean and Middle East by 2050 (Lelieveld et al., 2014).

O_3 is toxic to plants, some being more sensitive than others (Ashmore, 2005, Mills et al., 2016). Wheat is one of the most O_3 sensitive crops, with losses of global wheat yield estimated at 9.9% in the northern hemisphere [MILLS ET AL 2018] ~~(Van Dingenen et al., 2009~~) and it is suggested that until global O_3 pollution can be controlled the only adaptation available is the development of O_3 tolerant cultivars (Teixeira et al., 2011). Previous research assessed the O_3 sensitivity of several of the genome donors of modern bread wheat, with plants grown for 21 days and exposed to 100 ppb O_3 in open top chambers (Biswas et al., 2008a). *Ae. tauschii* and *T. monococcum* were found to be most sensitive to O_3, with reductions in shoot and root biomass, and rates of photosynthesis. The DD genome of *Ae. tauschii* was proposed as the primary source of wheat's O_3 sensitivity (Biswas et al., 2008a). Wild plants are known to be as sensitive to O_3 as crops (The Royal Society, 2008) and a recent study of the effect of O_3 on Natura 2000 grassland habitats in Europe revealed that areas in the Mediterranean are most at risk as a result of high levels of O_3 flux in the region (Harmens and Mills, 2016). Separate studies have confirmed changes in species composition due to a loss of O_3 sensitive species (Calvete-Sogo et al., 2016).

In the base rich soils of the Mediterranean, N deposition tends to lead to eutrophication, supporting the growth of nitrophilous species (Bobbink et al., 2010) with a subsequent impact on community composition. In Mediterranean grasslands, species richness has been found to decline in response to N deposition, with perennial grasses replacing forb species, especially those adapted to low N environments such as legumes (Bonanomi et al., 2013). In the Mediterranean and Near East, N deposition is already exceeding critical loads for some species (Phoenix et al., 2006), and climate change is predicted to increase other abiotic stress factors through increased temperatures and water shortages (Bonanomi et al., 2013, Intergovernmental Panel on Climate Change, 2013).

Despite both N deposition and O_3 pollution frequently occurring together, relatively little research has been performed on the effect on plants of the two pollutants in combination (Ochoa-Hueso et al., 2011, Sánchez-Martín et al., 2017). The research that has been done to date suggests that they do not necessarily replicate the effect of the individual pollutants and the outcome of interactions can differ depending on a variety of factors, including the plant's sensitivity to each pollutant (Mills et al., 2016). This study examined the responses of four of wheat's close wild relatives and one wheat cultivar at an early stage of development to N fertilisation and O_3 exposure, both separately and in combination. The study aimed to test the following hypotheses: firstly, that the growth of each species would be positively affected by N and negatively affected by O_3; and secondly, that there would be an interaction between N and O_3 resulting in N either ameliorating or exacerbating the effect of O_3 depending on the species.

Materials and methods

Plant establishment

Two primary and two secondary wild relatives of wheat, along with one wheat cultivar, were selected for the trial (Table 1). *Ae. tauschii*, *T. dicoccoides* and a close relative of *Ae. speltoides* are the likely genome donors of bread wheat (Marcussen et al., 2014). *T. monococcum* is one of the earliest forms of cultivated wheat (Matsuoka, 2011). All are annual species. De-husked seeds were germinated in an incubator set at 23.5 °C and sown into 1l plastic pots containing 650 g of Levington's John Innes No.1, a low nutrient seedling compost, with two plants per pot. Treatments were replicated four times. All plants were grown on in a temperature-controlled glasshouse (20.5 ± 2°C, relative humidity 51 ± 13 %) under natural daylight for 23 days (25 April – 18 May 2016), with pots rotated randomly every 3 - 4 days. Throughout the trial, all pots were manually watered, daily.

Nitrogen and ozone treatment

Prior to the start of O_3 exposure, 50 kg ha^{-1} yr^{-1} (25 ml) of ammonium nitrate solution was added to N treatment pots in two split doses to reduce leaching potential, on the 21st and 23rd days after planting (DAP). An equivalent amount of water was added to the remaining pots. At 24 DAP all pots were moved to closed top-lit chambers measuring 0.7 m x 0.7 m (24-h photoperiod, 200 ± 14 µmol m^{-2} s^{-1}, 24 ± 1.0 °C, 35.0 ± 6.4% humidity) in a temperature controlled glasshouse. Pots were divided by treatment, with 20 pots placed in each chamber. All pots were rotated and randomised within the chamber every two days and between chambers every four days, with two out of the three available air or O_3 chambers occupied at any one time. All chambers were supplied with charcoal filtered air from the glasshouse, with half the chambers ventilated continuously with O_3 using a *Triogen* lab O_3 generator throughout the treatment period of 21 days (19 May – 9 June 2016). The mean O_3 level was 32 ± 4 ppb in the air chambers and 113 ± 22 ppb across all three O_3-ventilated chambers, with a range of means from 104 ± 20 ppb to 126 ± 25 ppb, monitored using an *Aeroqual* v500 portable indoor air quality monitor. The length of trial and experimental conditions were similar to previous studies assessing the effect of O_3 on wheat cultivars and wheat's wild relatives (Biswas et al., 2008a).

Post-harvest measurements

Measurements of electrolyte leakage were obtained using a 2 cm tissue sample taken from one of the two plants in each pot at the point of harvest. Samples were placed in 1 ml of RO water, incubated at room temperature and shaken for 24 h. Conductivity was measured, after 24 h of shaking, and then again following freezing and autoclaving using a *Horiba* B-173 conductivity meter.

The plant not used for electrolyte tissue tests was harvested from each replicate pot with the shoot cut at the surface of the substrate. Measurements of shoot height, fresh shoot biomass, and leaf area (Transparent Belt Conveyor Li-3050A, *Li-Cor*, Lincoln, NE, USA) were taken immediately. Shoot biomass was determined by drying at 60°C for 4-6 days until a constant dry weight was reached. Root samples were retained *in situ* in pots and left to dry for at least 3 days before roots were washed by wet sieving and then dried in drying cabinets at 60°C for 4-6 days until a constant dry weight was reached. As the roots of the two plants in each pot were intertwined the total root dry biomass was halved to derive the root dry biomass data for each replicate and for the root:shoot ratio calculations. Two of the Triticum species, the cultivar *T*. 'Cadenza' and *T. dicoccoides,* had formed immature seed heads by the end of the trial period, though before any grain fill had occurred. These seedheads were cut at the base and dry seed head biomass measurements taken.

Shoot C:N ratios were obtained by grinding in a mixer mill (*Retsch* MM400, Hann, Germany), and analysed using a *Vario* EL III CHNOS Elemental Analyzer (Elementar Analysensysteme GmbH, Germany).

Statistical analysis

The effects of the different treatments on the parameters measured were assessed using Analysis of Variance (ANOVA). All data were checked for normality and log transformed where necessary. 3-way ANOVAs of the effects of N and O_3 and their interaction across all species were carried out for each variable. 2-way ANOVAs were carried out on the data for individual species and treatments. All statistical analyses were carried out using IBM SPSS statistics v22. Significance was assessed at $p < 0.05$.

Results

Effects of nitrogen and ozone on growth parameters

The height and leaf area of the five species (Fig. 1) were all significantly different to each other ($p = < 0.001$) under control conditions. There were no significant effects of the N or O_3 treatments on the height or leaf area of either *T. monococcum* or *Ae. tauschii*. N had a significant positive effect on the height of *Ae. speltoides* ($p = 0.011$) and a strong trend towards a positive interaction between N and O_3 ($p = 0.05$) whereby the O_3-only treatment reduced the height of *Ae. speltoides* whilst the N+O_3 treatment increased it. The leaf area of *T.* 'Cadenza' increased significantly in response to O_3 ($p = 0.026$), whilst there was a trend towards a positive effect of N on the leaf area of *T. dicoccoides* ($p = 0.067$).

There were significant differences between species' shoot and root biomass and subsequent root:shoot ratios ($p = < 0.001$) under control conditions (Fig. 2). There were no significant effects of the treatments on shoot biomass. There were trends toward a negative effect of O_3 on the shoot biomass of *Ae. tauschii* ($p = 0.057$), and an interaction between N and O_3 ($p = 0.067$) reducing the shoot biomass of *Ae. tauschii* further under the N+O3 treatment. There was also a small but non-significant positive response to N on the shoot biomass of *Ae. speltoides* ($p = 0.073$). None of the treatments had a significant effect on the root biomass of any species apart from *T.*'Cadenza' where O_3 had a significant negative effect ($p = 0.037$).

There was a significant difference in the seed head biomass (Fig. 3) between the two species which produced seed heads, *T.* 'Cadenza' and T. dicoccoides ($p = < 0.001$). *T.* Cadenza's seed head biomass was significantly reduced by O_3 ($p = 0.007$). There were no significant effects of any of the treatments on the seed head biomass of *T. dicoccoides*.

There were significant differences in the C:N ratios between species under control conditions (Fig. 4). There were no significant effects of N or O_3 alone, although the C:N ratios of *T.* 'Cadenza' ($p = 0.054$) and *T. monococcum* ($p = 0.061$) showed small but non-significant reductions with the addition of N. However, across the majority of species the combined N+O_3 treatment significantly reduced the C:N ratio ($p = 0.004$).

Effects of nitrogen and ozone on ozone injury

High variability in the electrolyte ratio data for most species meant there were no significant differences between species (Fig. 4) and there were only non-significant differences in the response to treatments. A significant statistical correlation was found between the electrolyte ratio and root dry biomass ($r^2 = -.322$, 2-tailed significance $p = 0.004$), with an increase in electrolyte leakage correlated with a reduction in root biomass.

Discussion

Ozone sensitivity and nitrogen responsiveness

Although *T. monococcum* showed some O_3 sensitivity, as has been found previously (Biswas et al., 2008a), none of the treatments had any significant effects on this species. *Ae. tauschii* and *Ae. speltoides* showed O_3 sensitivity but each responded differently to N and to N+O_3. *Ae. tauschii* had reduced shoot and root biomass in O_3-only and N+O_3 treatments (Fig.2), replicating similar responses to N+O_3 found in Mediterranean annual pastures (Calvete-Sogo et al., 2016). These results support those from a previous study on the O_3 sensitivity of *Ae. tauschii* where it was found to have reduced shoot and root biomass, and, as the donor of the DD genome, was proposed as the likely source of bread wheat's O_3 sensitivity (Biswas et al., 2008a). In addition, this new trial has shown that whilst *Ae. tauschii* was largely unresponsive to the sole addition of N, N exacerbated the negative effect of O_3, with further reductions in the shoot dry biomass of this species in response to the N+O_3 treatment; a response pattern also replicated in the wheat cultivar (Fig. 2).

The O_3 sensitivity of *Ae. speltoides* had not previously been assessed prior to this study. It was found to be both less affected by O_3 and more responsive to N than *Ae. tauschii*, with an ameliorating effect of N under the N+O_3 treatment on its height, leaf area, shoot biomass and electrolyte ratio (Figs. 1, 2, & 4). These results suggest that if O_3 occurs with N deposition, *Ae. speltoides* would be much less affected by O_3 than *Ae. tauschii.* In Mediterranean annual pastures similar effects have been found, with N moderating the effect of O_3 on biomass and yield, though not under high O_3 exposure (Calvete-Sogo et al., 2014).

Diploid *Aegilops* species, like *Ae. speltoides* and *Ae. tauschii*, are known to have a higher frequency of stomata compared to triploid species (Kharazian, 2007), potentially leading to higher levels of O_3 flux, especially with the large leaf area characteristic of *Ae. tauschii*. Whilst rapid growth caused by N deposition confers an advantage on nitrophilous species, rapid growth and high stomatal frequency is likely to be a disadvantage for plants also subject to O_3 pollution, unless this is accompanied by rapid detoxification of O_3 (Ashmore, 2005). These factors may make fast-growing annual species which are not nitrophilous, such as *Ae. tauschii*, potentially more vulnerable to O_3, especially when this rapid growth takes place in spring when high O_3 levels can occur (Thwaites et al., 2006, Calvete-Sogo et al., 2014, Calvete-Sogo et al., 2016).

N deposition is also known to have a variable effect on different species depending on their sensitivity to the oxidised or reduced forms of N (Stevens et al., 2011, van den Berg et al., 2016). In a study assessing the effect of ambient O_3 on the assimilation of both reduced and oxidised forms of N in immature wheat plants, O_3 increased the incorporation of reduced N, in the form of ammonium, whilst having no effect on the incorporation of oxidised N, in the form of nitrate (Möcker et al., 1996). This suggests that, where N and O_3 co-occur, the increase in reduced N levels, which results from O_3 exposure, could either be beneficial, or detrimental, depending on the sensitivity of the species to reduced forms of N.

Other studies have shown that O_3 also affects the structure and functioning of microbial communities in the rhizosphere (Zak et al., 2007, Li et al., 2013, Wu et al., 2016, Sánchez-Martín et al., 2017). Following long term exposure to 60 ppb O_3, O_3 sensitive wheat cultivars had significantly reduced microbial communities, including mycorrhizal fungi, compared to O_3 tolerant cultivars. This led to significant reductions in both reduced and oxidised N cycling soil processes, resulting in higher ammonia concentrations in the rhizosphere (Wu et al., 2016). Microbial communities associated with O_3 tolerant cultivars also showed a preference for easily degradable carbon sources whilst those associated with O_3 sensitive cultivars preferred more complex ones (Bao et al., 2015). A separate study on the different microbial communities associated with wheat and its wild relatives found that *Ae. tauschii* generates high levels of mycorrhizal fungi in its rhizosphere, and its DD genome appears to control the abundance of mycorrhizal fungi in both bread wheat, *T. aestivum*, and also synthetic wheat (Turner et al., 2013, Poole, 2016). Further study is required to assess the genetic links between wheat's wild relatives, its O_3 sensitivity and its mycorrhizal fungal communities, as well as to determine whether wheat's wild relatives have a preference for reduced or oxidised N.

Seed head production

Both the cultivar, *T*. 'Cadenza', and the wild relative, *T. dicoccoides,* produced immature seed heads within the 6-week time period of the trial, with this rapid generation of seed head biomass made possible by the use of a continuous photoperiod, a system approved by some wheat breeding programmes in order to speed up the research and breeding process (Hickey et al., 2009, Watson et al., 2018). Although further studies would be needed to assess responses under a natural lighting regime, both species were therefore exposed to O_3 during the time they flowered and developed seed heads, the growth stages when wheat is known to be at its most sensitive to O_3 (Soja et al., 2000, Ashmore, 2005). *T.* 'Cadenza' produced less seed head biomass compared to *T. dicoccoides,* and this was also significantly reduced by O_3 (-39%) and further reduced by N+O_3 (-52%), with both shoot and root biomass, and root:shoot ratio also reduced (Fig. 2). In comparison the seed head biomass of *T. dicoccoides* was not significantly affected by either O_3 (+11%) or N+O_3 (+0.5%) (Fig. 3). Of the five species, *T. dicoccoides* had the smallest leaf area (Fig. 1) and its root biomass was the least affected by treatments. It has also been found to have lower levels of mycorrhizal fungi compared to *Ae. tauschii* and *T. aestivum* (Poole, 2016). All these characteristics may contribute towards its O_3 tolerance, and may be of interest to plant breeders, especially as the O_3 sensitivity of wheat cultivars has been found to have increased over time. Experimental studies on both immature (Barnes et al., 1990, Velissariou et al., 1992, Biswas et al., 2008b) and mature wheat plants (Pleijel et al., 2006, Harmens et al., 2017) have shown that highly bred genotypes from the late 20th century and early 21st century are more O_3 sensitive compared to older ones. However, further study would be required to assess the specific cause of the O_3 tolerance of *T. dicoccoides* found in this trial.

None of the other three wild species developed seed heads within the time period available for this trial, and longer trials growing all species to seed ripened stage would be needed for a full assessment of their responses to O_3, especially after anthesis (Soja et al., 2000). However, any negative effects of these pollutants on their growth may also affect their ability to produce seed. O_3 pollution is known to affect both quantity and viability of seed in some species (Leisner and Ainsworth, 2012), and can also lead to changes in the timing of phenology (Sanz et al., 2011). In annual Mediterranean pastures many species store photosynthates early in the season for use in the drought-prone summer periods when flowering and seed production occur. If the O_3 exposure occurs at this early stage of plant growth then later seed production could be affected (Sanz et al., 2011).

Implications for the conservation of wheat's wild relatives

Due to the differing sensitivities of plant species, N deposition and O_3 lead to changes in the composition of communities in Mediterranean-type ecosystems (Ochoa-Hueso et al., 2011, Sanz et al., 2014, Calvete-Sogo et al., 2016). The results of this study suggest that the *in* *situ* populations of all four wild species may be affected by either N or O_3, with varying effects on each species when these pollutants co-occur. Further field study is required on the plant communities which host wheat's wild relatives to assess how N deposition and O_3 pollution are affecting these species and their competitors. In addition, as legume species in particular can be negatively affected by both N and O_3 (Sanz et al., 2014, Calvete-Sogo et al., 2016) an assessment of the impact of N and O_3 on the crop wild relatives of important leguminous crops such as pea, lentil and clover, also found in this Mediterranean and Near East region would be beneficial.

Implications for wheat breeding

Whilst all the species in this study have adequate numbers of accessions within international gene-banks (Castañeda-Álvarez et al., 2016), and as a result have been given low priority status for *ex situ* collecting purposes (Global Crop Diversity Trust, 2016), their *in situ* populations and genetic diversity remain important resources for plant breeding, both now and in the future. As in previous studies (Biswas et al., 2008a), *Ae. tauschii* was shown to be particularly O_3 sensitive, raising concerns that this trait could be transferred to some wheat lines through the plant breeding process, with *Ae. tauschii* having been used, for example, to improve disease resistance and drought tolerance (Global Crop Diversity Trust, 2016). In addition, the creation of synthetic hexaploid wheat by combining *T. turgidum* L. with *Ae. tauschii* is said to be a breakthrough in wheat breeding with the potential, as a result of its improved genetic diversity, to increase both yields and resistance to biotic and abiotic stresses (Li et al., 2014, Cox et al., 2017). It will be important, however, to ascertain whether synthetic wheat carries *Ae. tauschii's* O_3 sensitivity.

Conclusions

The *in situ* populations of crop wild relatives are of considerable economic value because they have the potential to supply the genetic diversity and resilience needed in the most important crops, including wheat. The potential impact of N deposition and O_3 on the conservation of wheat's wild relatives is therefore of concern, and an understanding of their N and O_3 sensitive traits is also important if they are to be used for breeding new wheat lines. *Ae. tauschii*, previously identified as the likely source of wheat's O_3-sensitivity, along with the cultivar *T*. 'Cadenza', were found to be O_3-sensitive, and their O_3 sensitivity was increased by the addition of N. *Ae. speltoides* proved to be nitrophilous, with N ameliorating the negative impact of O_3. Whilst the cultivar had significantly reduced seed head biomass in response to both O_3 and N+O_3 treatments, *T. dicoccoides* demonstrated O_3 tolerance in its root, shoot and seed head biomass.

The *in situ* populations of all four wild relatives in this study are likely to be affected by N and O_3, and, as N deposition and O_3 pollution affect species differently, the composition of the plant communities in which these crop wild relatives reside are also likely to be affected. Field studies would be needed to assess the extent of these changes and the implications there may be for other crop wild relatives in this region, especially legumes which are known to be particularly sensitive to both N and O_3. The results of this study can also inform the use of these wild relatives in future plant breeding, not only to enable use of their beneficial traits, but also to alert breeders to the inadvertent inclusion of N and O_3 sensitivity into wheat cultivars.

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