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Title: Acute and chronic ozone exposure temporarily affects seed germination in alpine plants

Thomas Abeli[a], Daniela B. Guasconi[a], Andrea Mondoni[a], Daniele Dondi[b], Antonio Bentivoglio[b], Armando Buttafava[b], Paolo Cristofanelli[c], Paolo Bonasoni[c], Graziano Rossi[a], Simone Orsenigo[d]

[a] Department of Earth and Environmental Sciences, University of Pavia, 27100 Pavia, Italy

[b] Department of Chemistry, University of Pavia, 27100 Pavia, Italy

[c] National Research Council of Italy, Institute for Atmospheric Sciences and Climate, 40129 Bologna, Italy

[d] Dipartimento di Scienze Agrarie e Ambientali - Produzione, Territorio Agroenergia, University of Milan, 20122, Milan, Italy

Corresponding author: Dr. Thomas Abeli, Department of Earth and Environmental Sciences, University of Pavia, Via S. Epifanio 14, 27100 Pavia, Italy. E-mail: thomas.abeli@unipv.it

Author Contributions: AT, SO and GR conceived the idea, AT, DBG, SO and AM conceived, designed and performed the experiments on seed germination, DD, A. Bentivoglio and A. Buttafava performed the EPR analysis, PC and PB provided the technical assistance for the production of ozone and wrote part of the introduction and methods. AT and DBG analyzed the data and wrote the manuscript with SO and AM.

Abstract

This study was the first to investigate the direct effects of anomalous concentrations of ozone mediated by summer heat waves on seed germination in alpine plants. During germination, the seeds were exposed to three peaks of O_3 concentration (125ppb for 5 and 10 days; 185ppb for 5 days), derived from measurements taken close to the species growing site. High O_3 concentration delayed the First Germination Time, increased the Mean Germination Time and reduced the Germination Percentage during and immediately after the treatment, but, in most cases, effects were weak and had almost vanished three weeks after the treatments. In a few cases, chronic exposure to O_3 (125 for 10 days' treatment) enhanced seed germination compared to the control, suggesting that ozone may induce antioxidant and DNA-repair mechanisms or dormancy-breaking effects in hydrated seeds. Although seed mortality increased during O_3 treatments in four species, the effect of O_3 on seed germination is mostly limited to the period of exposure, indicating that it is unlikely to produce permanent negative effects on seeds, during the germination phase. Our results show that the direct effect of O_3 on seeds of alpine plants may have minor impacts on plant reproductive performance during seed germination.

Keywords: Climate change; Electronic Paramagnetic Resonance; Extreme events; Pollution

Introduction

Stratospheric ozone (O_3) is a natural constituent of the atmosphere that plays a role in protecting the Earth's surface from an excessive amount of UV-B rays. Nevertheless, it is a greenhouse gas in the troposphere and a major air pollutant at ground level, especially at high concentrations (Black et al. 2000; UNEP & WMO 2011). In the troposphere, O_3 is produced by photochemical reactions in the presence of favourable concentrations of precursors like nitrogen oxides (NO_2 and NO_3), methane (CH_4), carbon monoxide (CO) and volatile organic compounds (VOC). Intensive O_3 production events occur more frequently during the warm season due to meteorological conditions that are favorable to the accumulation of O_3 precursors, like high temperature and solar irradiance, (The Royal Society 2008; Gilge et al. 2010). Thus, local patterns of temperature, sunlight and humidity affect the formation of O_3 at ground level, and peaks in the concentration of this gas are observed in correspondence to summer heat-waves (HWs) (Cristofanelli et al. 2015). At high concentrations, O_3 becomes an important ecological factor, which often negatively affects wild plants, crops and human health (UNEP & WMO 2011; Booker et al. 2009). Damages to vegetation usually occur over the threshold of 40 ppb (Bergmann et al. 1999; The Royal Society 2008), but during summer HWs, concentrations of over 100 ppb have been recorded (The Royal Society 2008). These concentrations may produce strong leaf injuries and disrupt photosynthetic metabolism (Betzelberger et al. 2010). O_3 may induce damages like visible or microscopical injuries on leaves (chlorosis or senescence), negatively affecting photosynthesis and plant growth in sensitive species

(Berrang et al. 1989; Timonen et al. 2004). Interestingly, ozone-mediated injuries on green structures are greater with warmer temperatures, as a consequence of changed physiological processes in the leaves, suggesting an interaction between O_3 and temperature (Albertine & Manning 2009). Plant reproductive performance is also strongly affected by O_3, which produces significant reductions of crop yields (Davison & Barnes 1998; Leisner & Ainsworth 2012). Specifically, damages to reproductive structures involve a decrease in pollen germination, tube growth and seed production, with more severe effects during flowering and seed maturation (Gerosa et al. 2009). Most of the experiments to test the effect of O_3 on seeds have involved their exposure during development and/or maturation on the mother plants (e.g. Stewart 1998; Landesmann et al. 2013), so the effect of O_3 on seeds through maternal influence is well-known. O_3 has been reported to have a marginal effect on seed maturation (Stewart 1998), germination, dormancy (Landesmann et al. 2013) and other seed attributes like protein or oil contents (Black et al. 2000), with plant response mostly depending on species, duration and intensity of the exposure. After harvesting, O_3 had weak negative effects on seed germination percentage in wheat (Savi et al. 2014) and a strong effect on seed macronutrient content in legumes (Mohamed et al. 1995; Iriti et al. 2009). Interestingly, little is known about the direct effect of anomalously high concentrations of O_3 caused by extreme heat waves. The current increases in frequency and intensity of HWs is often associated with periods of critical O_3 concentrations (European Environmental Agency 2014; Wittig et al. 2009), so the effects of this gas are likely to become very important, especially for the reproductive performance of alpine plants, which are highly sensitive to extreme weather events, like heat waves (Orsenigo et al. 2014).

In this study, we tested the direct effect of a heat-wave-mediated peak in O_3 concentration on seeds of nine alpine plant species during germination. Alpine plant seeds are known to mainly germinate in spring after snowmelt, when the soil moisture is high and air temperature increases rapidly (Körner 2003). Hence, the possibility that early summer HWs and consequent anomalous O_3 concentrations may affect seed germination cannot be ruled out. The effect of ozone during heat

waves may also interact with anomalous warm temperatures, but this interesting interaction was not considered here.

Additionally, we explored the relationship between ozone exposure and radical formation through the Electron Paramagnetic Resonance (EPR) spectrum. We aim to answer the following research questions: 1) do peaks of O_3 concentration affect seed germination parameters? 2) Does seed response to O_3 depend more on concentration or on the duration of exposure? 3) Is the seed germination response to O_3 permanent? 4) Is the seed germination response to O_3 reflected at physiological level?

Material and Methods

Species and seed collection

Seeds belonging to nine species (Table 1) were collected in the area around the Global GAW Meteorological Station "O. Vittori", 'ICO-OV' at Mt. Cimone (Northern Apennines, Italy), at Mt. Prado-Cusna (some 30 km apart) and in the Dolomites (Eastern Alps, Italy), between 1800 and 2300 m a.s.l. The species we collected were chosen either because of their abundance near the meteorological station that provided the data on ozone concentration, or because their germination requirements were well known (see Mondoni et al. 2011). Moreover, the responses to ozone of some of the species (i.e. *Silene acaulis*, *Festuca rubra*) and/or some congenerics (*Plantago*, *Silene*, *Vaccinium*) had already been studied, so it was possible for us to make comparisons with the literature (e.g. Stewart 1998; Hayes et al. 2007).

The seeds were collected at the time of their natural dispersal, in August-September 2013 (Hay & Smith 2003). Seed germination was immediately tested on fresh seeds, before their storage in the Seed Bank at the University of Pavia, under standard seed banking conditions (15% R.H., 15°C). The ozone treatments were carried out between May and June 2014. The germination percentage between fresh and stored seeds at the time of the experiment was the same for both pooled data

(Mann-Withney z = -0.347; n = 54; p = 0.729) and single species data, but the difference was poorly significant for *Silene acaulis*. Storage conditions should therefore not have affected seed germination.

Experimental design

Seeds from all nine species were exposed to three O_3 treatments. Firstly, they were exposed to 125ppb for 5 days (hereafter 125_5), simulating the peak of O_3 concentration recorded at the 'ICO-OV' station between 21st and 25th July 2006. Although this period may not represent the optimum seed germination timing for alpine plants under natural conditions (usually occurring earlier in summer), we took this event as a case study because it represents one of the highest concentrations of O_3 ever recorded in the Alps and Northern Apennines in the decade 2003-2012 (see World data Center for Greenhouse Gases: http://ds.data.jma.go.jp/gmd/wdcgg/). Furthermore, similar O_3 concentrations were recorded in more suitable periods for alpine plant germination, such as in June 2003 (118.2 ppb), June 2005 (110.1 ppb) and June 2006 (110.2 ppb). Then, in order to test whether the effect of O_3 would be higher under prolonged exposure or under higher concentration of the gas, we performed two additional treatments consisting of a 10-day exposure to 125 ppb (125_10) and a 5-day exposure to 185 ppb (185_5), respectively. The value of 185 ppb was recorded in the lowland of northern Italy in summer 2006 (European Environmental Agency 2007). O_3 concentrations recorded at the ICO_OV station during the considered heat wave were almost constantly higher than 100 ppb, with peaks higher than 115 ppb at night (from hourly data; Figure 1), so exposure at the above mentioned O_3 concentrations lasted 24 hours a day for all treatments throughout the experiment. This avoided the problem of considering variations in O_3 concentration between night and day. After the ozonization, seed germination continued in the same conditions as in the control treatment.

Seed germination experiment and ozone treatment

Seed germination tests involved sowing three replicates of 20 seeds each on 1% distilled water–agar held in Petri dishes. Treatments were carried out in temperature and light-controlled incubators using a 12-h daily photoperiod (photosynthetically active radiation 100 μmol · m^{-2} · s^{-1}) and alternating temperatures of 20/13°C. The temperature of 20°C corresponded to the average maximum daily air temperature recorded at the ICO-OV Station during the 2006 HW (21^{st} – 25^{th} July), while 13°C was the average minimum daily air temperature. Seeds under these germination test conditions were exposed to a control (ambient air with the measured concentration of ozone ranging between 0 and 1 ppb) and to different O_3 concentrations (see above).

A glass bell positioned inside the incubator was used as an ozonization chamber to avoid the reaction between O_3 and the plastic of the incubator. A constant concentration of O_3 was generated by an O_3 calibrator 1008-PC (Dasibi Environmental Corporation, Glendale, CA, USA) and insufflated through a Polytetrafluoroethylene (PTFE) pipe (diameter 0.4 mm) into the glass bell. Another pipe of the same diameter acted as discharge and guaranteed the flux of O_3.

Glass Petri dishes were positioned on steel shelves within the bell and their position was changed every day in order to assure uniform conditions during the O_3 treatment.

Germination test and recorded parameters

During the incubation, all seeds were checked for radicle emergence at daily intervals throughout O_3 exposure (five or ten days according to the treatment), then at weekly intervals until the end of each test (four weeks) and germinated seeds were removed. At the end of the test, ungerminated seeds were first sown on Agar + GA_3, then dissected with a scalpel under a microscope to determine whether they were viable or not. A comparison of the number of dead seeds (i.e. moldy seeds) in the control and O_3 treatments revealed that O_3 affected seed viability (see results section);

hence, mouldy seeds were not removed from the total when calculating the germination percentage, as they were considered as an effect of the treatment. In other words, dead seeds were included in the germination percentage. The following parameters were recorded: 1) First Germination Time (FGT), or days elapsed from seed sowing to the first germination event for each species, 2) seed Germination Percentage (GP), and 3) Mean Germination Time (MGT). MGT was calculated using the formula:

$$MGT = \sum_i^n, n_i t_i \ / \ N$$

where n_i is the number of seeds that germinated at the time t_i, t_i is the number of days between the beginning of the germination test and seed scoring, and N is the total number of seeds that germinated. In order to infer differences in seed GP and MGT between treatments at different time intervals, analysis was performed on data at the end of the O_3 exposure (five or ten days from sowing, according to the treatment) and at the end of the germination tests, after 28 days from sowing (hereafter referred to as end of the test).

Characterization of Electron Paramagnetic Resonance (EPR) spectrum

EPR spectra are related to free radical species which can easily be detected, even at low concentrations (few ppm or less). The EPR spectrum of treated 125_10 and non-treated seeds was measured in a subset of four species (*F. rubra* subsp. *commutata*, *P. alpina*, *S. suecica* and *S. acaulis*) selected on the preliminary results of the treatment 125_5. The most affected species in the former treatment were selected for the EPR spectrum. EPR measurements were performed on days 1, 6 and 11 from seed sowing and on dry seeds for technical reasons (humidity masks the radical signal), thus on seeds in a glassy state. The EPR instrument used for the measurements was a Bruker, EMX, X-band continuous wave spectrometer equipped with an EPR cavity Bruker ER4119HS. The seeds were inserted into the EPR quartz tube (5 mm external diameter, 4 mm internal diameter) without exceeding 3 cm in height, which corresponds to the region of maximum

sensitivity of the cavity. This is the equivalent of a sample weight in the range 200 - 600 mg. Two different experimental setups were used, as described below. The first setup was used in order to enhance the region of carbon and oxygen centered radicals. In particular, EPR acquisition parameters were fixed as follows: magnetic field centered at 3345.2 G with a sweep width of 100 G; modulation amplitude and frequency were 2 G and 100 kHz, respectively; microwave power was 10.06 mW with a frequency of 9.4 GHz. A full spectrum was obtained by accumulating 2 µ-scans. The second setup was used for the determination of metals (iron and manganese). In this case, the EPR acquisition parameters were fixed as follows: magnetic field centered at 2373.9 G with a sweep width of 3000 G; modulation amplitude and frequency were 2 G and 100 kHz, respectively; microwave power was 10.06 with a frequency of 9.4 GHz. A full spectrum was obtained by accumulating 5 µ-scans. The signals obtained were normalized according to the number of scans, and the receiver gain and weight, using the following formula:

$$\frac{H(peak)}{receiver\ gain\ \times n^{\circ}\ \mu - scans\ \times weight}$$

where *H* is the height of the peak and *n* is the number of micro-scans.

The analysis of the EPR spectrum was associated to a measurement of the seed water content through the TGA 1 STARe System model (Mettler, Toledo). The gas flow rate (nitrogen) was 4 L/min and the temperature program started at 25 °C up to 100 °C with a heating rate of 5 °C/min followed by a constant temperature of 100 °C for 60 minutes.

Data analysis

Mann-Whitney tests on pooled data per treatment and on each species were used to compare FGT and MGT between control and O_3 treatments. A non-parametric test was chosen based on the number of replicates per treatment (3). The effect of O_3 treatments on seed germination was analyzed with logistic regressions performed on each species, in which the dependent (binary)

variable was the germinated/non-germinated seeds and the descriptor was the treatment (control vs. O_3). Differences between O_3 treatments were analyzed using several Mann-Whitney tests performed on each species. Changes in the time of the EPR spectrum, and thus in the radical species within the seeds were analyzed in control and exposed seeds. To do so the normalized EPR peaks (expressed in arbitrary units) were regressed against the duration time of the treatment in each of the four species tested for this parameter. Regressions were performed on log10 transformed data.

Results

First Germination Time (FGT)

The number of days to the first seedling emergence was significantly affected in all O_3 treatments. Overall, O_3 exposure delayed germination in all treatments compared to the control, with the long exposure treatment 125_10 having the greatest effect (Table 2). However, at species level, we did not detect a consistent species response, with some species delaying germination in a given treatment but not in others (Table 2).

Seed mortality

The lowest seed mortality was recorded in the control test (1.37 ± 1.24; mean ± st. dev.), while the highest mortality was found in the treatment 125_5 (2.81 ± 3.051). Significant differences between treatments for seed mortality were found in: *A. alpinus, F. rubra* subsp. *commutata*, *S. acaulis* and *S. nutans* (Table 3).

Seed Germination Percentage

Control vs. 125 ppb for 5 days

Seed germination at the end of O_3 exposure significantly differed between O_3 treatments and the control in *A. clavennae*, *F. rubra* subsp. *commutata*, *P. alpina*, *S. acaulis* and *S. suecica* (Table 4; Figure 2). *V. myrtillus* had not germinated by the end of the treatment (5 days), so it was excluded from the analysis. At the end of the test (28 days from sowing), the germination of seeds that experienced O_3 was reduced in *F. rubra* subsp. *commutata, F. violacea* subsp. *puccinellii, P. alpina* and *S. nutans* (Table 4; Figure 3).

Control vs. 185 ppb for 5 days

Germination at the end of O_3 exposure significantly reduced in *F. rubra* subsp. *commutata, F. violacea* subsp. *puccinellii, S. acaulis* and *S. suecica* (Table 4; Figure 2). As with the other treatments, *V. myrtillus* did not germinate within 5 days, so it was excluded from the analysis. At the end of the test, differences between control and treatment were significant for *A. clavennae* and *V. myrtillus* (Table 4; Figure 3), but in opposite ways: while the treatment stimulated germination in *A. clavennae* (+ 45.8%), it reduced germination in *V. myrtillus* (-20.3%; Table 4; Figure 2).

Control vs. 125 ppb for 10 days

At the end of O_3 exposure, a significant effect of O_3 was found in *P. alpina*, *S. acaulis, and S. suecica* (Table 4). As with the 125(5) treatment, *V. myrtillus* did not germinate within 10 days, so it was excluded from the analysis. At the end of the germination test, differences between control and treatment were still significant for *P. alpina* and *S. suecica* (Table 4, Figure 3), but in this case GP increased (+ 33.9% and + 13.8%, respectively).

Mean Germination Time (MGT)

Control vs. 125 ppb for 5 days

Overall, MGT was not affected by the O_3 treatment either at the end of the O_3 exposure (Mann-Whitney Z -1.365; n = 16; P = 0.172), or at the end of the test (Mann-Whitney Z -1.369; n = 18; P = 0.171). However, MGT increased at the end of O_3 exposure (Table 5) in *A. clavennae*, *A. alpinus* and *S. suecica* by 1.1 (± 1.0), 0.2 (± 0.1) and 0.8 (± 0.06) days (mean ± st. dev.), respectively. At the end of the germination test, the effect of O_3 on MGT was still present in *A. clavennae*, *S. acaulis* and *S. suecica*, increasing by 3.2 (± 2.6), 5 (± 0.84) and 2.2 (± 0.78) days, respectively (Table 5).

Control vs. 185 ppb for 5 days

Overall, MGT did not differ between control and treatment either at the end of O_3 exposure (Mann-Whitney Z = -1.683; n = 16; P = 0.092) or at the end of the experiment (Mann-Whitney Z = -0.309; n = 18; P = 0.757). At the end of O_3 exposure, MGT significantly differed between control and treatment in *F. rubra* subsp. *commutata, P. alpina* and *S. suecica*. MGT significantly increased by 0.6 (± 0.2) and 1.4 (± 1.1) days in *F. rubra* subsp. *commutata* and *S. suecica*, respectively (Table 5). In contrast, MGT reduced by 0.5 (± 0.3) days in *P. alpina*. At the end of the test, MGT significantly increased by 2.9 (± 1), 10.3 (± 6.6) and 1.7 (± 1) days in *A. clavennae*, *S. acaulis* and *S. suecica*, respectively (Table 5). MGT significantly reduced by 2.7 (± 1.9) days in *V. myrtillus*.

Control vs. 125 ppb for 10 days

Overall, MGT at the end of O_3 exposure significantly increased by 1.25 (± 1.3) days in seeds exposed to O_3 (Mann-Whitney Z = -2.417; n = 16; P < 0.05). MGT significantly increased in *F. rubra* subsp. *commutata, S. nutans* and *S. suecica* by 3.4 (± 1.3), 0.4 (± 0.2) and 2 (± 0.2) days, respectively (Table 5). At the end of the germination test, overall MGT did not significantly differ between the control and the treatment (Mann-Whitney Z = -1.723; n = 18; P = 0.085). However,

poorly significant differences at P = 0.05 were found in *A. clavennae*, *A. alpinus*, *F. rubra* subsp. *commutata*, *P. alpina*, *S. acaulis* and *S. suecica* (Table 5).

EPR spectrum

We observed three different areas that corresponded to iron (III), manganese and organic radicals (carbon and oxygen centered). The variations of inorganic species (iron and manganese) during the process were negligible, so we focused on carbon and oxygen species. The intensity (i.e. the concentration of radicals) of the organic radicals showed correlations with both water content (as determined by TGA) and duration of exposure to O_3. The seeds treated with 125 ppb of O_3 showed a clear increase of organic radicals with increasing treatment duration (Figure 4). These increases were significant in *F. rubra* subsp. *commutata* ($R = 0.986$; $df = 3$; $P = 0.014$), *P. alpina* ($R = 0.994$; $df = 3$; $F = 155.229$; $P < 0.01$) and *S. suecica* ($R = 0.969$; $df = 3$; $F = 30.808$; $P < 0.05$), but not in *S. nutans*. No significant increases of organic radical with time were detected in control seeds.

Discussion

This study investigated the direct effects of anomalous concentrations of O_3 on seeds of alpine plants during germination in order to understand whether heat-wave-mediated peaks in the concentration of this gas may affect plant reproduction. Indeed, heat waves are often related to anomalous concentrations of O_3, known to have negative effects on plant leaf and reproduction (Leisner & Ainsworth 2012). However, only a few studies on crop species have considered the direct effects of O_3 on dry seeds (e.g. Ciccarese et al. 2007; Marique et al. 2012) and investigated even less its effect on seeds during germination.

Here we have shown that O_3 has direct and contrasting effects on seeds of alpine plants during germination that differ between species, gas concentration and duration of exposure. FGT was

significantly delayed in five species (Table 2) and MGT was increased in six species compared to control (Table 2, 4). However, such a germination delay was not consistent among treatments within species (e.g. in *V. myrtillus* an increase in MGT was found only in treatment 185_5; Table 5) and there was generally a great variability between the replicates, indicating that the effect of O_3 on the timing of germination cannot be generalized. Such different responses to O_3 across different species and treatments (increase or decrease in GP and MGT, depending on species and exposure) were observed in seeds exposed to O_3 before the germination test (Bosac 1992; Steward 1998; Black et al. 2000). A greater and more durable effect on MGT was observed under a chronic exposure (treatment 125_10), but results were only poorly significant.

In our study, a greater effect of O_3 was found on seed GP, mostly during the period of exposure. For example, considering the three ozone treatments, seed GP reduced in six species (Figure 2). Moreover, an increasingly negative effect with increasing O_3 concentration (i.e. from treatment 125_5 to 185_5) was found in *F. rubra, F. violacea* and *S. suecica*, although it did not reach significance. A progressively higher effect of O_3 with its increased concentration was also described in wheat (Feng et al. 2008) and, similarly, Landesmann et al. (2013) found decreasing seed germination in *Asperula arvensis* when O_3 concentration was increased from 90 to 120 ppb. Furthermore, our results show that at the end of the germination tests (i.e. 4 weeks after sowing), the effects of O_3 on seeds were not as clear as right at the end of the exposure (i.e. 5-10 days after sowing). Six species (*A. clavennae*, *P. alpina*, *S. suecica*, *F. rubra* subsp. *commutata, F. violacea* subsp. *puccinellii* and *V. myrtillus*) were still affected but without a clear pattern across species and treatments (Figure 3). For example, in *A. clavennae*, *P. alpina* and *S. suecica*, at least one O_3 treatment increased seed GP (Figure 3). Conversely, the two *Festuca* species, *S. nutans* and again *P. alpina,* were highly negatively affected by the treatment 125_5. Surprisingly, the genus *Festuca* and *Plantago* were almost insensitive to O_3 when the effect on the aboveground biomass was considered (see e.g. *Festuca* spp. and *Plantago lanceolata* in Hayes et al. (2007)). In the other species, the effect of O_3 vanished a few days after the treatment (e.g. *S. acaulis*).

Although high concentrations of O_3 increased seed mortality in some species, as found here in *A. alpinus*, *F. rubra* subsp. *commutata*, *S. nutans* and *S. acaulis*, our observations suggest that the effect of O_3 on seeds during germination is transient and limited to the period of exposure. Furthermore, the weak delay of FGT and the increased MGT indicate that O_3 does not produce strong, permanent negative effects on seed germination in alpine plants. In further support to this indication, the O_3 concentrations tested here are among the highest recorded in southern Europe over a ten-year period and refer to data taken 2 m above the soil surface, according to standard atmospheric measurements. More detailed O_3 monitoring at soil surface is needed to better understand whether the concentrations of this gas vary at the level of seeds after dispersal, and if so, in what way. Unfortunately, O_3 dynamics and concentrations in the soil have been poorly investigated (but see Turner et al. 1973; Wesely et al. 1981).

On one hand, our results are in accordance with the role of seeds in plant reproduction. Unlike seedlings, seeds are highly resistant to environmental stress in order to guarantee the survival of the next generation. The reduced/delayed germination under O_3 prevents damages due to the direct effect of O_3 on seedlings, thus reducing the loss of progeny (e.g. Prozherina et al. 2009). On the other hand, our findings open new questions on the role of seed relative humidity in response to O_3-mediated oxidative stress. In fact, the transient effect found in our experiment may be related to the scavenging activity and the activation of antioxidant mechanisms in fully-hydrated seeds during germination (Ventura et al. 2012). This may explain the increased GP found in *P. alpina* and *S. suecica* and the reduced seed mortality in *A. alpinus*, *F. rubra* subsp. *commutata* and *S. nutans* under the effect of 125_10. However, the antioxidant metabolism has high energetic costs, and this may also explain the difference in seed mortality between species and treatments. Moreover, we should consider that seed quality is influenced by several factors related to the health of the mother plants, environmental conditions during seed maturation, etc. (Mondoni et al. 2014). These external factors may have affected seed response to the ozone. This unexpected behavior highlights the potential role of O_3 in seed repair ability, which may have interesting applications in the seed

industry when dealing with both crops and wild plants (see e.g. NASSTEC project; www.nasstec.eu). However, at the time of maturation and dispersal, seeds may have a lower moisture content, which may prevent the activation of reactive oxygen species mediated signaling mechanisms to counteract the oxidizing properties of O_3. Further research on this interesting aspect should be performed.

Free radical involvement in seed response to O_3 is clearly shown by the EPR spectra: they demonstrate that O_3 increased the concentration of radicals (carbon and oxygen species) in all tested species, except *S. nutans*. However, Ciccarese et al. (2007) found no differences in seed germination between dry and imbibed seeds of wheat, barley and peas treated with ozone. Going back to our research questions, we can conclude that although O_3 affects the seed germination of alpine plants at a physiological level, these effects are species-specific, weak and transient, even at exceptional O_3 concentrations. Considering that warm temperatures recorded during HWs may enhance seed germination if water is available (Orsenigo et al. 2015), the contrasting interaction between ozone and temperature should be further investigated to consider, for example, O_3 concentrations at soil level, a parameter that is rarely available. Finally, further research is still needed to clarify the potential use of ozone as a seed priming technique.

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References

Aeschimann D, Lauber K, Moser DM, Theurillat J-P. 2004. Flora Alpina, Zanichelli, Bologna.

Albertine JM, Manning WJ. 2009. Elevated night soil temperatures result in earlier incidence and increased extent of foliar ozone injury to common bean (*Phaseolus vulgaris* L.). Environ Pollut 157; 711–713.

Bergmann E, Bender J, Weigel H-J. 1999. Ozone threshold doses and exposure–response relationships for the development of ozone injury symptoms in wild plant species. New Phytol 144: 423 – 435.

Berrang P, Karnosky DF, Bennett JP. 1989. Natural selection for ozone tolerance in *Populus tremuloides*: field verification. Can J For Res 19: 519-522. Doi:10.1139/x89-080.

Betzelberger AM, Gillespie KM, Mcgrath JM, Koester RP, Nelson RL, Ainsworth EA. 2010. Effects of chronic elevated ozone concentration on antioxidant capacity, photosynthesis and seed yield of 10 soybean cultivars. Plant Cell Environ 33: 1569–1581.

Black VJ, Black CR, Roberts JA, Stewart CA. 2000. Impact of ozone on the reproductive development of plants. New Phytol 147: 421–447.

Booker F, Muntifering R, McGrath M, Burkey K, Decoteau D, Fiscus E, Manning W, Krupa S, Chappelka A, Grantz D. 2009. The ozone component of global change: potential effects on agricultural and horticultural plant yield, product quality and interactions with invasive species. J Integr Plant Biol 5: 337–351. doi: 10.1111/j.1744-7909.2008.00805.x.

Bosac C. 1992. The impact of ozone and sulphur dioxide on reproductive development in oilseed rape. PhD dissertation, University of Nottingham.

Ciccarese F, Sasanelli N, Ciccarese A, Ziadi T, Mancini L. 2007. Seed disinfestation by ozone treatments. In: Proceedings of the IOA Conference and Exhibition, Valencia, Spain, 2007.

Cristofanelli P, Scheel H-E, Steinbacher M, Saliba M, Azzopardi F, Ellul R, Fröhlich M, Tositti L, Brattich E, Maione M, Calzolari F, Duchi R, Landi TC, Marinoni A, Bonasoni P. 2015. Long-term

surface ozone variability at Mt. Cimone WMO/GAW Global Station (2165 m a.s.l., Italy). Atmos Environ 101: 23-33.

Davison AW, Barnes JD. 1998. Effects of ozone on wild plants. New Phytol 139; 135–151.

European Environmental Agency. 2007. Air pollution by ozone in Europe in summer 2006. Overview of exceedances of EC ozone threshold values for April–September 2006. EEA Technical report No 5/2007.

European Environmental Agency. 2014. Air pollution by ozone across Europe during summer 2013. Overview of exceedances of EC ozone threshold values: April–September 2013. EEA Technical report No 3/2014.

Feng Z, Kobayashi K, Ainsworthz EA. 2008. Impact of elevated ozone concentration on growth, physiology, and yield of wheat (*Triticum aestivum* L.): a meta-analysis. Global Change Biol 14: 2696–2708. doi: 10.1111/j.1365-2486.2008.01673.x.

Gerosa G, Marzuoli R, Rossini M, Panigada C, Meroni M, Colombo R, Faoro F, Iriti MA. 2009. Flux-based assessment of the effects of ozone on foliar injury, photosynthesis and yield of bean (*Phaseolus vulgaris* L. cv. Borlotto Nano Lingua di Fuoco) in open-top chambers. Environ Pollut 157; 1727–1736. Doi 10.1016/j.envpol.2008.06.028.

Gilge S, Plass-Duelmer C, Fricke W, Kaiser A, Ries L, Buchmann B, Steinbacher M. 2010. Ozone, carbon monoxide and nitrogen oxides time series at four alpine GAW mountain stations in central Europe. Atmos Chem Phys 10: 12295–12316.

Hay FR, Smith RD. 2003. Seed maturity: when to collect seeds from wild plants. In: Smith RD, Dickie JD, Linington SH, Pritchard HW, Probert RJ editors. Seed Conservation: Turning Science into Practice: Royal Botanic Gardens, Kew. pp. 97–133.

Hayes F, Jones MLM, Mills G, Ashmore M. 2007. Meta-analysis of the relative sensitivity of semi-natural vegetation species to ozone. Environ Pollut 146: 754-762. doi:10.1016/j.envpol.2006.06.011.

Iriti M, Di Maro A, Bernasconi S, Burlini N, Simonetti P, Picchi V, Panigada C, Gerosa G, Parente A, Faoro F. 2009. Nutritional traits of bean (*Phaseolus vulgaris*) seeds from plants chronically exposed to ozone pollution. J Agr Food Chem 57: 201–208.

Körner C. 2003. Alpine plant life, 2nd edn. Springer, Berlin.

Landesmann JB, Gundel PE, Martínez-Ghersa MA, Ghersa CM. 2013. Ozone exposure of a weed community produces adaptive changes in seed populations of *Spergula arvensis*. PlosOne 8: E75820.

Leisner CP, Ainsworth EA. 2012. Quantifying the effects of ozone on plant reproductive growth and development. Global Change Biol 18: 606–616. doi: 10.1111/j.1365-2486.2011.02535.x.

Marique T, Allard O, Spanoghe M. 2012. Use of self-organizing map to analyze images of fungi colonies grown from *Triticum aestivum* seeds disinfected by ozone treatment. Inter Microbiol: Article ID 865175. doi:10.1155/2012/865175.

Mohamed AI, Bhardwaj HL, Rangappa M, Hoggard G. 1995. Seed characteristics and nutrient composition of selected beans (*Phaseolus vulgaris*) with different ozone tolerance. Plant Food for Human Nutrition 47: 29–38.

Mondoni A, Orsenigo S, Donà M, Balestrazzi A, Probert R, Hay FR, Petraglia A, Abeli T. 2014. Environmentally induced transgenerational changes in seed longevity: maternal and genetic influence. Ann Bot 113: 1257-1263. Doi:10.1093/aob/mcu046.

Mondoni A, Probert RJ, Rossi G, Vegini E, Hay FR. 2011. Seeds of alpine plants are short lived: implications for long-term conservation. Ann Bot 107: 171–179. Doi: 10.1093/aob/mcq222

Orsenigo S, Abeli T, Rossi G, Bonasoni P, Pasquaretta C, Gandini M, Mondoni A. 2015. Effects of autumn and spring heat waves on seed germination of high mountain plants. PlosOne in press. Doi: 10.1371/journal.pone.0133626

Orsenigo S, Mondoni A, Rossi G, Abeli T. 2014. Some like it hot and some like it cold, but not too much: plant responses to climate extremes. Plant Ecol 215: 677-688. doi:10.1007/s11258-014-0363-6.

Prozherina N, Nakvasina E, Oksanen E. 2009. Impact of experimentally elevated ozone on seed germination and growth of Russian pine (*Pinus sylvestris*) and Spruce (*Picea* spp.) provenances. Ambio 38: 443-447.

Savi GD, Piacentini KC, Bittencourt KO, Scussel VM. 2014. Ozone treatment efficiency on *Fusarium graminearum* and deoxynivalenol degradation and its effects on whole wheat grains (*Triticum aestivum* L.) quality and germination. J Stored Prod Res 59: 245-253.

Stewart CA. 1998. Impact of ozone on the reproductive biology of *Brassica campestris* L. and *Plantago major* L. Doctoral Thesis Doctor of Philosophy of Loughborough University, May 1998.

The Royal Society. 2008. Ground-level ozone in the 21st century: future trends, impacts and policy implications. RS Policy document 15/08. London.

Timonen U, Huttunen S, Manninen S. 2004. Ozone sensitivity of wild field layer plant species of northern Europe. A review. Plant Ecol 172: 27-39.

Turner NC, Reich S, Waggoner PE. 1973. Removal of Ozone by soil. J Environ Qual 2: 259-264.

UNEP, WMO. 2011. Integrated Assessment of Black Carbon and Tropospheric Ozone. UNEP, Nairobi, 2011.

Ventura L, Donà M, Macovei A, Carbonera D, Buttafava A, Mondoni A, Rossi G, Balestrazzi A. 2012. Understanding the molecular pathways associated with seed vigor. Plant Physiol Bioch 60: 196-206. doi: http://dx.doi.org/10.1016/j.plaphy.2012.07.031.

Wesely ML, Cook DR, Williams RM. 1981. Field measurement of small ozone fluxes to snow, wet bare soil, and lake water. Bound-Lay Meteorol 20: 459-471.

Wittig VE, Ainsworth EA, Shawna W, Naiduz L, Karnosky DF, Stephen P. 2009. Long quantifying the impact of current and future tropospheric ozone on tree biomass, growth, physiology and biochemistry: a quantitative meta-analysis. Global Change Biol 15: 396–424. doi: 10.1111/j.1365-2486.2008.01774.x

Table 1 Taxa selected for the experiment with collecting location, flowering time (according to Aeschimann et al. 2004) and the chorotype.

Family	Species	Locality	Flowering Time (months)	Chorotype
Asteraceae	*Achillea clavennae* L.	P.so Fedaja	6-8	E-Alps/Illyric
Asteraceae	*Aster alpinus* L.	Mt. Cusna	6-8	Arctic-Alpine
Poaceae	*Festuca rubra* L. subsp. *commutata* (Gaudin) Markgr.-Dann	Mt. Cimone	7-8	Circumboreal
Poaceae	*Festuca violacea* subsp. *puccinellii* (Parl.) Foggi, Graz.Rossi & Signorini	Mt. Cusna	7-8	Endemic
Plantaginaceae	*Plantago alpina* L.	Mt. Cimone	5-8	Orophitic-European
Cariophyllaceae	*Silene acaulis* (L.) Jacq.	P.so Fedaja	6-9	Circumboreal
Cariophyllaceae	*Silene nutans* L.	Mt. Cimone	5-8	Circumboreal
Cariophyllaceae	*Silene suecica* (Lodd.) Greuter & Burdet	Mt. Prado	7-8	Circumboreal
Ericaceae	*Vaccinium myrtillus* L.	Mt. Cimone	4-7	Circumboreal

Table 2 First Germination Time. Delays in the number of days to first emergence in three ozone treatments compared to the control. Significant differences between the control and the other ozone treatments are highlighted in bold.

Species	Treatment	z	df	Delay (days) ± st. dev.	P
A. clavennae	125_5	-2.121	5	1.7 (1.1)	**< 0.05**
A. clavennae	185_5	-1.581	5	0.7(0.6)	n.s.
A. clavennae	125_10	-2.121	5	1.3 (0.6)	**< 0.05**
A. alpinus	125_5	-1.650	5	1.0(1.0)	n.s.
A. alpinus	185_5	-1.650	5	1.0(1.0)	n.s.
A. alpinus	125_10	-1.650	5	1.0(1.0)	n.s.
F. rubra	125_5	-1.000	5	-0.6(1.15)	n.s
F. rubra	185_5	-2.236	5	1.0 (0.0)	**< 0.05**
F. rubra	125_10	0.000	5	0.0 90.0)	n.s.
F. violacea	125_5	-1.000	5	-0.3(0.6)	n.s.
F. violacea	185_5	-1.000	5	0.3(0.6)	n.s.
F. violacea	125_10	-2.236	5	1.0 (0.0)	**< 0.05**
P. alpina	125_5	-1.581	5	0.7(0.6)	n.s.
P. alpina	185_5	-1.581	5	0.7(0.6)	n.s.
P. alpina	125_10	-1,792	5	2.3(1.1)	n.s.
S. acaulis	125_5	-0.696	5	1.383.5)	n.s.
S. acaulis	185_5	-0.913	5	0.7(1.5)	n.s.
S. acaulis	125_10	-1,650	5	1.7(1.5)	n.s.
S. nutans	125_5	-1.000	5	0.3(0.6)	n.s.
S. nutans	185_5	-1.000	5	0.3(0.6)	n.s.
S. nutans	125_10	-2.236	5	1.0 (0.0)	**< 0.05**
S. suecica	125_5	-2.236	5	2.0 (0.0)	**< 0.05**
S. suecica	185_5	-2.121	5	1.3 (0.6)	**< 0.05**
S. suecica	125_10	-0.696	5	1.08(2.6)	n.s.
V. myrtillus	125_5	-1.000	5	-0.7(1.1)	n.s.
V. myrtillus	185_5	-2.236	5	2.0(0.0)	**< 0.05**
V. myrtillus	125_10	-2.236	5	3.0(0.0)	**< 0.05**

Total	125_5	-2.110	47	0.71 (1.5)	**< 0.05**
Total	185_5	-3.301	47	0.87 (0.9)	**< 0.01**
Total	125_10	-4.125	47	1.17 (1.2)	**< 0.001**

Table 3 Seed mortality. Percentage seed mortality in three ozone treatments and comparison of the treatments. Significant differences between the ozone treatments are highlighted in bold.

	Percentage mortality (mean ± st. dev.)				Kruskal-Wallis		
	Control	125_5	185_5	125_10	$\chi 2$	df	P
A. clavennae	0.33(0.58)	1.33(0.58)	1.67(2.08)	3.00(2.65)	2.781	3	n.s.
A. alpinus	1.67(0.58)	2.67(0.58)	1.00(1.00)	0.00(0.00)	8.500	3	**<0.05**
F. rubra	0.67(0.58)	7.67(2.89)	4.00(2.64)	0.00(0.00)	9.454	3	**<0.05**
F. violacea	0.67(0.58)	4.67(5.51)	2.33(1.15)	1.67(1.15)	4.171	3	n.s.
P. alpina	3.33(0.58)	3.67(3.05)	5.33(3.05)	2.00(1.00)	2.850	3	n.s.
S. acaulis	1.67(1.15)	0.67(0.58)	1.67(0.58)	12.33(1.53)	8.274	3	**<0.05**
S. nutans	0.00(0.00)	1.33(0.58)	0.67(0.58)	0.00(0.00)	8.567	3	**<0.05**
S. suecica	1.67(1.53)	0.33(0.58)	0.00(0.00)	0.67(0.58)	4.056	3	n.s.
V. myrtillus	2.33(1.15)	3.00(2.65)	1.33(0.58)	2.33(2.31)	1.428	3	n.s.

Table 4 Results for the germination percentage at the end of the ozone exposure (left panel) and at the end of the germination tests (right panel). Results from logistic regressions show the effect of the three ozone treatments on seed germination percentage with respect to the control treatment. Significant differences between the control and the ozone treatments are highlighted in bold.

Species	**End of O_3 exposure**						**End of test**					
	125_5		**185_5**		**125_10**		**125_5**		**185_5**		**125_10**	
	$\chi^2_{(1)}$	**P**	$\chi^2_{(1)}$	**P**	$\chi^2_{(1)}$	**P**	$\chi^2_{(1)}$	**P**	$\chi^2_{(1)}$	**P**	$\chi^2_{(1)}$	**P**
A. clavennae	5.804	**<0.05**	1.455	n.s.	1.649	n.s.	0.035	n.s.	13.828	**<0.001**	0.575	n.s.
A. alpinus	2.245	n.s.	3.746	n.s.	0.372	n.s.	0.324	n.s	1.047	n.s.	0.000	n.s.
F. rubra	11.152	**<0.001**	33.986	**<0.001**	0.001	n.s.	21.011	**<0.001**	4.256	n.s.	2.889	n.s.
F. violacea	1.985	n.s.	12.079	**<0.001**	0.203	n.s.	9.079	**<0.01**	1.228	n.s.	0.459	n.s.
P. alpina	5.739	**<0.05**	1.200	n.s.	22.986	**<0.001**	6.625	**0.01**	0.034	n.s.	5.345	**<0.05**
S. acaulis	12.257	**<0.001**	6.733	**<0.05**	6.008	**<0.05**	2.683	n.s.	0.846	n.s.	0.320	n.s.
S. nutans	2.750	n.s.	0.184	n.s.	2.806	n.s.	4.184	**<0.05**	0.366	n.s.	1.395	n.s.
S. suecica	44.253	**<0.001**	67.437	**<0.01**	6.942	**<0.01**	0.690	n.s.	0.240	n.s.	7.944	**<0.01**
V. myrtillus							1.655	n.s.	4.092	**< 0.05**	0.148	n.s.
Total												

Table 5 Mean Germination Time expressed in days (mean ± st. dev.) for the control and the ozone treatments. Significant differences between the control and the ozone treatments are highlighted in bold. Z values from the Mann-Whitney test are reported. Degrees of freedom are 5 in all cases.

Species								
End of O_3 exposure								
	C	**125_5**	**$Z_{(125\_5)}$**	**185_5**	**$Z_{(185\_5)}$**	**C_10**	**125_10**	**$Z_{(125\_10)}$**
A. clavennae	4.60(0.17)	**5.67(1.15)**	**-2.023**	4.89(0.19)	-1.573	6.40(0.34)	6.90(0.41)	-1.528
A. alpinus	4.61(0.06)	**4.93(0.11)**	**-2.023**	4.83(0.29)	-0.674	5.86(1.34)	7.13(0.30)	-1.528
F. rubra	4.35(0.17)	4.23(0.60)	-0.218	**5.00(0.00)**	**-2.087**	3.21(1.51)	**6.63(0.40)**	**-1.964**
F. violacea	4.37(0.37)	4.57(0.25)	-0.886	4.83(0.14)	-1.107	5.25(0.83)	6.59(0.38)	-1.528
P. alpina	4.12(0.24)	3.50(0.50)	0.127	**3.64(0.13)**	**-1.964**	5.18(0.84)	5.83(2.25)	-0.218
S. acaulis	4.67(0.29)	5.17(2.56)	0.817	4.56(0.51)	0.000	5.53(0.65)	5.83(0.76)	-0.443
S. nutans	4.15(0.17)	4.38(0.37)	0.376	4.32(0.13)	-1.091	4.70(0.23)	**5.14(0.07)**	**-1.993**
S. suecica	4.22(0.06)	**5.00(0.00)**	**-2.087**	**5.67(1.15)**	**-1.993**	4.46(0.29)	**6.56(0.12)**	**-1.993**
End of germination tests								
	C	**125_5**	**$Z_{(125\_5)}$**	**185_5**	**$Z_{(185\_5)}$**	**125_10**	**$Z_{(125\_10)}$**	
A. clavennae	9.74(1.47)	**12.91(1.38)**	**-1.964**	**12.60(1.03)**	**-1.964**	**13.77(1.53)**	**-1.964**	
A. alpinus	8.00(1.04)	9.15(1.56)	-1.107	7.27(0.43)	-1.107	**10.15(0.70)**	**-1.964**	
F. rubra	7.29(0.25)	7.35(0.30)	-0.886	7.00(0.00)	-1.549	**8.45(0.90)**	**-1.964**	
F. violacea	8.00(1.32)	8.43(0.56)	-0.655	7.68(0.29)	-0.218	7.93(0.41)	-0.218	
P. alpina	9.17(1.03)	9.50(2.18)	-0.655	10.28(1.33)	-0.655	**14.03(0.49)**	**-1.964**	
S. acaulis	8.05(0.93)	**13.00(1.73)**	**-1.993**	**18.40(6.08)**	**-1.964**	**12.35(1.44)**	**-1.964**	
S. nutans	7.35(0.35)	7.52(0.45)	-0.899	7.46(0.50)	-0.443	7.47(0.20)	-0.471	
S. suecica	7.28(0.24)	**9.46(0.98)**	**-1.964**	**8.95(0.92)**	**-1.964**	**10.68(0.17)**	**-1.964**	
V. myrtillus	19.50(1.13)	19.59(0.33)	-0.655	**16.83(1.15)**	**-1.993**	19.00(1.20)	0.658	

FIGURE CAPTIONS

Figure 1 Hourly data of the concentration of ozone recorded by the Meteorological Station "ICO-OV" at Mt. Cimone during the summer heat wave in July 2006.

Figure 2 Germination percentage (mean ± 2S.E.) of each species at the end of the ozone exposure. Different letters indicate statistically significant differences of germination at $P < 0.05$ level.

Figure 3 Germination percentage (mean ± 2S.E.) of each species at the end of the germination test. Different letters indicate statistically significant differences of germination at $P < 0.05$ level.

Figure 4 Relationships between height of EPR peak and time of seed exposure to 125 ppb of O_3.