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Ambient levels of nitrogen dioxide (NO_2) may reduce pollen viability in Austrian pine (*Pinus nigra* Arnold) trees — Correlative evidence from a field study

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A R T I C L E I N F O

Article history:
Received 20 November 2007
Received in revised form
18 April 2008
Accepted 18 April 2008
Available online 9 June 2008

Keywords:
Austrian pine
Pollen viability
Nitrogen dioxide
TTC-test
Sampling design

A B S T R A C T

A fully randomized sampling design was adopted to test whether pollen viability of Austrian pine (*Pinus nigra* Arnold) was impacted by NO_2 pollution. Spatial strata (500*500 m each) with high (41.9–44.6 μg m^{-3}) and low (15.4–21.0 μg m^{-3}) NO_2 were selected from a defined population in a small area (236.5 km^2, <200 m range in elevation) in Northern Italy. Pollen viability was measured by means of the Tetrazolium (TTC) test. Analysis of variance by means of a generalised linear model showed that NO_2 was a significant factor ($P=0.0425$) affecting pollen viability. Within the treatment, no significant differences were detected among replicates. Within each replicate, sampling unit data were significantly different ($P=0.000$) and this suggested some improvement in the applied sampling design was needed. Pollen viability was significantly related to pollen germination ($P<0.01$) and tube length ($P<0.01$). This suggested a possible impact of NO_2 on the regeneration of Austrian pine in polluted environments.

1. Introduction

A number of actual and potential impacts of air pollutants are used at the international level to set thresholds to protect vegetation (UN/ECE, 2004). Most of them consider quantity and quality of yield (e.g. wheat and potato), visible injury (different crops, semi-natural vegetation, trees) and growth (forest trees) (UN/ECE, 2004). Much less attention is paid to other possible impacts that – in perspective – may have long-lasting effects on the ability of plants to reproduce and adapt to changing environmental conditions. For example, pollen grains have a potential in this respect (Wolters and Martens, 1987; Bellani et al., 1988; Iannotti et al., 2000; Black et al., 2000). Pollen can exhibit biochemical (Bist et al., 2004), ultrastructural (Stirban et al., 1984; Bellani et al., 1997), morphological (Cela Renzoni et al., 1990; Micieta and Murin, 1998) and physiological (Cox, 1988; Kristen, 1997) alterations due to exposure to anthropogenic compounds. Physiological alterations are generally tested by analysing pollen viability, which is the ability of pollen to complete post-pollination events and to realize fertilization. Pollen viability can be evaluated by means of several tests. The more common ones are based on the evaluation of *in vitro* germination and pollen tube growth, dehydrogenase activity (Tetrazolium Test, or TTC-test), esterase activity and membrane integrity (Fluorochromatic Reaction Test, or FDA-test) (Shivanna and Rangaswamy, 1992). Some authors have demonstrated a reduction in viability for pollen collected in differently polluted situations in contrast to pollen from control (unpolluted) areas (Houston and Dochinger, 1977; Cela Renzoni et al., 1990; Comtois and Schemenauer,

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doi:10.1016/j.scitotenv.2008.04.048

1991; Kormutak et al., 1994; Ostrolucka et al., 1995; Tretyakova et al., 1996; Kormutak, 1996; Tretyakova and Bazhina, 2000; Pukacki and Chalupka, 2003; Kormutak, 2004; Gottardini et al., 2004). Unfortunately, limited attention was paid in the past to ensure field studies adopting a formal statistical sampling design to compare groups of trees subjected to different pollution regimes; as a consequence, a study allowing a formal statistical inference about the estimated response parameter (i.e. the mean pollen viability) in relation to the stressor of concern (e.g. different pollution levels) is still lacking. This is a serious drawback because the strength of the conclusion of field studies relies for the most part on the adopted sampling design (Hurlbert, 1984; Eberhardt and Thomas, 1991). In addition, the lack of a proof-of-concept under "real world" conditions and with a formal design may limit the appeal of the approach and its application.

In the present study, we adopted a fully randomized sampling design to evaluate whether ambient levels of pollutants have an actual effect on pollen viability of trees. We considered the effects of nitrogen dioxide (NO_2) on pollen viability because NO_2 is reported to be the only primary gaseous phytotoxic pollutant of concern at the time of pollen ripeness in the study area (Provincia Autonoma di Trento, 1998). Actually, sulphur dioxide (SO_2) is no longer a concern in the area, and ground level ozone (O_3) concentrations are usually low during the spring time. Also, Non-Methane Hydrocarbons (NMHC) and airborne Particulate Matter (PM10) measured in the study area, show low concentrations in the period of concern (Piano provinciale di tutela della qualità dell'aria, Allegato F, http://www.dolomitez.com/Web_AGF/documentazione/aria/Allegati/ALLEGATO-F.pdf). The null hypothesis (H_o) was that trees located under low and high NO_2 pollution levels have similar pollen viability. In this perspective, pollen viability was tested by contrasting groups of trees subjected to significantly different NO_2 levels.

2. Materials and methods

2.1. Concept of the study and area of investigation

The study was designed to compare trees living in the field under fairly homogeneous conditions, but subjected to different NO_2 levels. For this purpose, "treatment" and "control" groups (see below) were identified according to NO_2 levels. Field observational studies are always subject to possible confounding factors which are difficult to control. To minimize their impact, we decided to incorporate the most important ones (environmental gradients, age and health condition of trees) into the design of the study (see below). Several actions were undertaken. Firstly, the study domain was restricted to a small area (236.5 km^2) with limited range in elevation (199–356 m a.s.l.) and with homogeneous climatic conditions. Secondly, we concentrated only on even-sized (size assumed to be an indicator of tree age) and healthy (no obvious discoloration and defoliation) Austrian pine trees. Austrian pine (*Pinus nigra* Arnold) is common in the study area, has abundant pollen production which is easy to collect and with known sensitivity to air pollutants (Keller and Beda, 1984; Micieta and Murin, 1998). Thirdly, we ensured replication of "treatments" and randomization within each "treatment" replicates (see Sections 2.2 and 2.3). This allowed a distribution of the treatment and control groups across the range of the target population. The number of replicates per treatment was decided on the basis of a pilot study (Gottardini et al., 2004) and to keep the study feasible, taking into account the restriction imposed by the limited time window for pollen collection and the huge amount of work needed at this step.

The study area was located near Trento, Northern Italy (Fig. 1). Despite all actions undertaken to minimize and control possible confounding factors, it is worth noting that, given the non-random nature of the NO_2 distribution over the study area (high levels occur close to urban areas; low levels occur in the countryside), it was not possible to achieve a complete factorial combination of treatments (Hurlbert, 1984). This suggests that a role for possible confounding factors may not be ruled out.

2.2. Pollution levels and no₂ NO_2 measurements

To identify suitable sites for our "treatment" – high NO_2 level- and "control" – low NO_2 level-groups, we proceeded as follows.

First, the whole study area was divided in 146 spatial strata of 500*500 m each (Fig. 1). Second, each spatial stratum was examined for the presence of healthy, adult Austrian pine trees. All in all, 41 spatial strata out of 146 were found to host at least 10 Austrian pine trees.

Third, these 41 strata were classified in relation to the expected NO_2 pollution level on the basis of their land-use (remote, rural and urban zone) and the occurrence of possible significant category of pollution sources within their boundaries (point, linear, areal sources and intensive farming). In practice, each spatial stratum received a score in relation to land-use (urban = 4; rural = 2; forest/remote = 1). The presence of significant pollution sources caused an additional score of 1 for each category identified. For example an urban area (score 4) with a power plant (score 1), a large traffic route (score 1) and an incinerator (score 1) received a score = 7. At the end, all strata were ranked between 1 (expected lowest pollution) and 8 (expected highest pollution). The eight spatial strata with the highest and the eight with the lowest scores were selected.

Fourth, in these 16 strata, NO_2 measurements by passive sampling were performed between 21 March and 18 May 2006. One passive sampler (PASSAM AG, see http://www.passam.ch/ for details) was located in an open site within each stratum. The sampler consisted of a polypropylene tube. NO_2 is collected by molecular diffusion along an inert tube to an absorbent (triethanolamine) and determined spectrophotometrically by the Saltzmann method (Saltzman, 1960). The samplers were placed in a special shelter to protect them from rain and minimize the wind influence. Results are reported in Fig. 2. The mean NO_2 concentration over the study area was 31.4 ± 7.21 $\mu g\ m^{-3}$ (confidence interval at $P = 0.01$). Based on these data, the four spatial strata characterized by lower NO_2 values (15.4–21.0 $\mu g\ m^{-3}$) were considered as "control" and the four spatial strata with higher NO_2 values (41.9–44.6 $\mu g\ m^{-3}$) as "treatment". Although not directly comparable to our two-month average, it is worth noting that the European Union directive 1999/30/CE set the threshold for human health protection at 40 $\mu g\ m^{-3}$ annual mean (http://eur-lex.europa.