

Histological, metabolomic, and transcriptomic differences in fir trees from a peri-urban forest under chronic ozone exposure

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Abstract

Urbanization modifies ecosystem conditions and evolutionary processes. This includes air pollution, mostly as tropospheric ozone (O₃), which contributes to the decline of urban and peri-urban forests. A notable case are fir (*Abies religiosa*) forests in the peripheral mountains southwest of Mexico City, which have been severely affected by O₃ pollution since the 1970s. Interestingly, some young individuals exhibiting minimal O₃—related damage have been observed within a zone of significant O₃ exposure. Using this setting as a natural experiment, we compared asymptomatic and symptomatic individuals of similar age (≤ 15 years old; $n = 10$) using histological, metabolomic and transcriptomic approaches. Plants were sampled during days of high (170 ppb) and moderate (87 ppb) O₃ concentration. Given that there have been reforestation efforts in the region, with plants from different source populations, we first confirmed that all analysed individuals clustered within the local genetic group when compared to a species-wide panel (Admixture analysis with ~1.5K SNPs). We observed thicker epidermis and more collapsed cells in the palisade parenchyma of needles from symptomatic individuals than from their asymptomatic counterparts, with differences increasing with needle age. Furthermore, symptomatic individuals exhibited lower concentrations of various terpenes (β -pinene, β -caryophyllene oxide,

α -caryophyllene and β - α -cubebene) than asymptomatic trees, as evidenced through GC-MS. Finally, transcriptomic analyses revealed differential expression for thirteen genes related to carbohydrate metabolism, plant defense, and gene regulation. Our results indicate a rapid and contrasting phenotypic response among trees, likely influenced by standing genetic variation and/or plastic mechanisms. They open the door to future evolutionary studies for understanding how O₃ tolerance develops in urban environments, and how this knowledge could contribute to forest restoration.

Introduction

Rapid urbanization has severely disturbed entire ecosystems since the beginning of the industrial age (Bai et al., 2017), raising the important questions of how species cope with human-transformed environments and which molecular, evolutionary and ecological processes are involved (Rivkin et al., 2019). It is regularly thought that for species to persist in urban areas, they must adapt rapidly (Johnson & Munshi-South, 2017). However, for adaptation to occur, selection needs to operate on heritable variation, which can determine whether a species persists or disappears from urban areas. Rapid adaptation seems particularly important for pollution tolerance, one of the strongest and most abrupt challenges that an urban species may face (Santangelo et al., 2018). This is especially challenging for long-lived species, such as forest trees, implying that adaptation must occur within a few generations or be complemented by plastic responses (Müller-Starck & Schubert, 2001). The genetic basis and plastic responses to pollution have been studied using a plethora of methods, from traditional provenance trials to genomic and transcriptomic analyses (Papadopoulos et al., 2020; Whitehead et al., 2017). However, most research has been done under controlled conditions, meaning that studies in natural settings are

needed for exploring the differential phenotypic responses in putatively tolerant versus sensitive individuals, and verifying if the same genes and pathways pinpointed in controlled studies can also be detected in the field.

One of the most common and harmful urban pollutants is tropospheric ozone (O_3), which is generated by photochemical reactions that involve by-products of fossil fuel burning (Churkina et al., 2017). Ozone is toxic to plants and has caused significant damage to forest ecosystems in and around heavily polluted cities (Ashmore, 2005; Cho et al., 2011). Given the key role that urban forests perform as providers of ecosystem services, understanding how O_3 tolerance operates in trees is a pivotal step for informing conservation and reforestation programs of degraded (peri-)urban forests. This requires field studies with an urban-ecology perspective, aiming to understand how O_3 tolerance develops and operates in natural settings, where tree responses to O_3 are also expected to be more complex and entangled with other sources of stress (Nunn et al., 2006).

In plants, O_3 damage, and the molecular mechanisms underlying the response to O_3 exposure, has been studied for over 20 years, using both field and laboratory experiments with controlled conditions (Felzer et al., 2007; Hayes et al., 2020). O_3 enters the plant through the stomata and triggers the formation of different reactive oxygen species (ROS), causing metabolic stress and resulting in cellular death, as ROS travel through the apoplast (Tausz et al., 2007). Several candidate genes have been postulated to cope with O_3 -mediated metabolic stress (e.g., Hayes et al., 2020). However, strategies seem to differ between species and among populations within species (Baier et al., 2005; Hasan et al., 2021; Ludwików & Sadowski, 2008). For instance, differential sensitivity to ozone has been documented between poplars from more polluted and less polluted areas in the USA, according to

both common garden and field experiments (Berrang et al., 1991). Furthermore, differential foliar damage (related to O₃ exposure) has been observed among sacred fir (*Abies religiosa*) provenances in central Mexico (Hernández-Tejeda & Benavides-Meza, 2015).

More than 5 million vehicles circulate daily in Mexico City (CDMX; INEGI, 2018), making it one of the most air-polluted cities in the world (ONU, 2018). Its geographic location, mostly enclosed within a high-elevation valley, and the high fossil fuel consumption generates perfect conditions for tropospheric O₃ formation and accumulation (Bravo-Alvarez & Torres-Jardón, 2002; Molina et al., 2019). For instance, while O₃ concentration in unpolluted air ranges between 20-50 ppb (Seinfeld, 1989), daily levels in CDMX continuously reached 200 ppb during the 1990s (SEDEMA, 2020; Fig. 1a). Such elevated values still persist as isolated peaks (reaching up to 180 ppb by 2017; SEDEMA, 2020; Fig. 1a), particularly between March and June, when temperatures in CDMX are the highest and precipitation the lowest (CONANP, 2006). Given that days with good air quality (*i.e.* <70 ppb) are still scarce (Fig 1a) and that O₃ maxima are still well above the tolerable thresholds for human and ecosystem health (NOM-020-SSA1-2104; SEDEMA Report, 2017), a constant selective force with strong episodic peaks, that coincide with the start of the growing season for most local plant species, is assumed to occur within the peri-urban forests of CDMX.

Atmospheric drainage in CDMX mostly occurs between the southwestern mountains, which are dominated by sacred fir forests (Fig. 1d; Alvarado-Rosales et al., 2017). There is an ongoing decline of these forests, associated with the detrimental effects of O₃ (de Bauer & Hernández-Tejeda, 2007), inadequate management, excessive water extraction and recurrent forest fires (Alvarado R.,

1989; Macías-Sámano & Cibrián-Tovar, 1989). Firs within these forests exhibit O₃ damage in the form of reddish needles, which are rich in phenolic compounds and have degraded vacuoles and disintegrated spongy and palisade parenchyma (Alvarado-Rosales & Hernández-Tejeda, 2002; Alvarez et al., 1998). Damage becomes visible in one-year-old needles, which die after the third year of exposure. When compared to unpolluted areas of the species' range, such damage often leads to decreased vigour and increased susceptibility to several pests (Alvarado-Rosales & Hernández-Tejeda, 2002; Hernández-Tejeda & Benavides-Meza, 2015).

Although previous studies have described O₃ damage symptoms and pointed to this pollutant as the main cause for fir forest decline in CDMX (Alvarado R., 1989; Alvarado-Rosales & Hernández-Tejeda, 2002; de Bauer & Hernández-Tejeda, 2007), little attention has been paid to phenotypic differences for O₃-related symptoms until recently (Hernández-Tejeda & Benavides-Meza, 2015), when some apparently healthy young plants were observed within a heavily damaged stand. Complementing these observations in one of the most polluted cities of the world with methodological approaches to examine the effect of O₃ on plants can improve our understanding of how O₃ tolerance develops and operates in natural settings. For instance, at the histological level, we could expect more cellular damage in symptomatic trees than in asymptomatic individuals. Similarly, a deficient regulatory response to the oxidative stress caused by O₃ can be translated in the differential accumulation of certain metabolites, like some specific terpenes that have been observed in asymptomatic plants from various species after ozone exposure (Miyama et al., 2019; Kopaczky et al., 2020). Lastly, transcriptomic analyses can help to narrow down the number of genes involved in the response to O₃ exposure

and to examine plastic responses in gene expression under varying levels of O₃ (DeBiasse & Kelly, 2016).

Here, we explored the differential histological, metabolomic (terpene) and transcriptomic responses to ozone pollution within a natural peri-urban forest dominated by *A. religiosa*. Given that previous reforestation attempts have been carried out in this zone, we first determined the geographic origin of individuals and then looked for differentially expressed genes between asymptomatic and symptomatic trees during days of high and relatively low ozone concentrations. This study represents a first step to guide peri-urban forest management from an eco-evolutionary perspective.

Material and methods

Study area and sampling

The study site is located near CDMX, in one of the most exposed areas to tropospheric ozone, the “Cruz de Coloxtitla” ravine, in the village of Santa Rosa Xochiac, next to the ‘Desierto de los Leones’ National Park (Alvarado-Rosales et al., 2017; Fig. 1d). We traced a quadrant of 80x137 m (19.285 N, -99.301 E; Fig. 2a) within this zone and focused on young (10-15 years old) *Abies religiosa* [(Kunth) Schlechtendahl et Chamisso] trees. We chose five plants exhibiting large numbers of reddish needles, indicative of damage by O₃ (Miller et al., 1994; hereafter referred to as “symptomatic” trees), as described elsewhere (Alvarado-Rosales & Hernández-Tejeda, 2002; Alvarez et al., 1998). Additionally, we selected five apparently healthy individuals, which had no visible damage in any branch (“asymptomatic” trees from hereon; Fig. 2-b, S2). Symptomatic and asymptomatic trees (n=10) were distributed heterogeneously within the zone and were separated by at least five meters from

each other (Fig. 2a). Needle samples were collected for each tree in three time points with contrasting O₃ concentration: moderate (April 15th, 2017; 87 ppb), intermediate (May 13-14th 2017, 120-94 ppb) and high (May 17th, 2017; 170 ppb; Fig. 1b-c), according to daily measurements from the nearest (PEDREGAL, PE) atmospheric station (available at <http://www.aire.cdmx.gob.mx/default.php?opc=%27a8Bhnml=%27&opcion=Zg==>). Needles were preserved in RNA Later and stored at -70°C until processing. The first sampling period roughly coincided with the start of the bud-burst period for this population (personal observations). Sampling was performed for all individuals between 13:30-15:30 hrs (Fig. 1c); needles were selected from three sections of the same branch, in six branches per individual. Each branch section corresponded to a particular growth period (*i.e.*, 2015, 2016 and 2017; Fig. 2b). No symptomatic individual had leaves more than three years old.

188

189 *Genotyping and geographic origin of tolerant trees*

Reforestation efforts in the study zone involved germplasm from foreign provenances (Hernández-Tejeda & Benavides-Meza, 2015). To verify that sampled plants originated locally, from natural regeneration, we employed previously published SNP data for 318 individuals from 19 populations of *A. religiosa* distributed across its natural range (Giles-Pérez et al., 2022). This data was used to assign the collected individuals to previously reported genetic clusters (Fig. 3a). To do so, we used 80 mg of needle tissue for DNA extraction using liquid nitrogen and the QUIAGEN DNeasy® Plant Mini Kit (cat. No. 69104), following the manufacturer's protocol. DNA integrity was checked in 1% agarose gel, and its concentration quantified with a Qubit™ v 3.0. Libraries were prepared following the protocol from

Poland & Rife (2012) after digestion with restriction enzymes *MspI* (C | CGG) and *PstI* (TGCA | G); a Pippin prep (SAGE sciences) was used to select the adequate fragment size before PCR amplification and sequencing. DNA sequencing was conducted in an Illumina's HiSeq2500 SE100 lane (100bp) and in a Nextseq lane (100 bp) were at the Institute of Integrative Biology and Systems at Université Laval, Canada (<http://www.ibis.ulaval.ca/en/services-2/genomic-analysis-platform/>). Read quality was examined using FastQC (<http://www.bioinformatics.braham.ac.uk/projects/fastqc/>) before and after demultiplexing and quality filtering. Reads were assembled *de novo*, and ipyrad was used for SNP calling (Eaton, 2014). Parameters used were: mindepth_statistical 8, mindepth_majrule 100000, clust_threshold 0.9. To optimize SNP calling, we followed the recommendations from Mastretta-Yanes et al. (2015), modified for ipyrad. We aimed keeping SNPs genotyped in at least 90% of individuals and with minor allele frequencies (MAF) above 0.05. Individuals with more than 10% missing data were discarded with PLINK1.9 (Purcell et al., 2007), and additional random individuals were removed until retaining only 3-5 trees of each population, along with the ten focus individuals of this study.

Pairwise relatedness between each pair of individuals within populations was calculated using PLINK 1.9 (Chang et al., 2015), as closely related individuals could bias further analyses, including population structure and assignment (Sethuraman, 2018). Only one of the focus (symptomatic) individuals was randomly discarded because of high relatedness ($r > 0.25$) with another symptomatic tree (Fig. S3). ADMIXTURE v 1.3.0 (Bhatta et al., 2019) was used to infer population structure by supposing between 1 and 5 genetic clusters (K); optimal K was assumed to be the one with smallest cross validation error (CV).

225

226 *Anatomical analyses*

227 Transverse histological sections were prepared for five needles per branch from
228 three branches of each tree, all sampled during the high O₃ concentration periods.
229 Following sampling, needles were embedded in distilled water according to Sandoval
230 et al. (2005) and cut in 7-10 mm sections. Sections were immersed overnight in a
231 fixative solution composed of 50% ethanol, 10% formaldehyde, 35% double distilled
232 water and 5% glacial acetic acid (FAA). After washing with distilled water and
233 dehydration in a graded terbutylic alcohol series, sections were embedded in
234 Paraplast™, by adding 12-15 flakes every 30 min in an oven at 58 °C, until doubling
235 the alcohol volume. Sections were stored at 56 °C for 3 weeks until forming solid
236 blocks (inclusion cubes), which were further sectioned with a rotating microtome
237 (American Optical 820; 12µm). Ten to 15 transversal tissue sections were obtained
238 per needle. The sections were first hydrated and dyed with safranin, then dehydrated
239 within a graded ethanol series and stained with dye fast green (FCF), using a
240 previously standardized method for sacred fir (Sandoval et al., 2005). Afterwards,
241 they were mounted on slides and dried for 15 days in an oven at 56° C. We looked
242 for cell structures previously reported as symptoms of O₃ damage (Fig. S2; Gimeno
243 & Ibars, 2009). Samples were photographed in an Axioskope Car Zeiss
244 photomicroscope for examining tissue-level damage, compared to a reference
245 description of *A. religiosa* (Alvarez et al., 1998).

246

247 *Terpenes analysis*

248 Two- and three year-old needles (corresponding to the growth years of 2015 and
249 2016) collected during moderate (87 ppb) and high (170 ppb) O₃ concentration

250 periods were used to quantify relative terpene abundances (Ibrahim et al., 2019).
251 Approximately 80-95 mg (fresh-weight) tissue preserved in liquid nitrogen was
252 macerated with a mortar and pestle with 2 mL of dichloromethane, transferred to
253 microtubes, and centrifuged (within tubes) for 1 min at 14,000 rpm. The supernatant
254 was recovered and dried with compressed air, and the pellet was resuspended in
255 450 µL of dichloromethane and 50 µL of 1 mg/mL 1-isopropylphenol (as internal
256 standard). After homogenization, 2 µL were injected into a gas chromatograph with a
257 Split/splitless injector (Agilent Technologies 6850 Network GC System) coupled to a
258 mass spectrometer (5975C VL MSD with Triple-Axis Detector) and a Xylan
259 (Quadrex) 30 m * 0.25 mm * 0.25 µm capillary column. Analyses were performed at
260 230°C in the splitless mode (3 min). The initial temperature was set at 70°C for 2
261 min, then increased to 230°C at a rate of 20° C / min, and maintained for 5 min.
262 Helium (*i.e.*, carrier gas) was injected at a rate of 1 mL / min; the temperatures of the
263 transfer line, ionization source, and quadrupole analyzer were 280°C, 230°C, and
264 150°C, respectively. Analyses were performed by electronic impact at 70 eV using
265 the full spectrum scan mode (SCAN). For relative quantification, peak areas were
266 integrated and normalized to the internal standard. Each peak (associated to a
267 specific metabolite) was validated according to its retention time and mass spectrum
268 based on the National Institute of Standards and Technology (NIST) library.

269 Only terpenes with similar fragmentation patterns or retention times (TR),
270 observed in at least 60 % of the samples and with at least 80% identification
271 probability were retained. A matrix of relative abundance per 100 g of tissue was
272 then generated for comparison between tree conditions (asymptomatic vs.
273 symptomatic), periods (high and moderate O₃), and needle age (2015, 2016; Fig. 1)
274 through a linear model using R (R Core Team, 2021), assuming a Gamma

275 distribution. We compared the goodness of fit of the models with the Akaike's
276 information criterion. The better model was Metabolites Concentration ~ Condition *
277 Period. We performed non-paired comparisons, with Wilcoxon tests, to explore
278 variations in metabolite composition between asymptomatic and symptomatic
279 groups, between periods (87 ppb vs 170 ppb) and needle ages (one year vs two
280 years). Analyses were performed in the stats package 4.1.2 (R Core Team, [2021](#))
281 and results were visualized with ggplot2 3.3.5 (Wickham, [2016](#)).

282

283 *Differential expression analyses*

284 One- and two-year old needles (2015 and 2016) sampled during the moderate (87
285 ppb) and high (170 ppb) O₃ concentration periods were further analyzed for
286 differential expression through RNA sequencing. Total RNA was isolated using a
287 Spectrum RNA Plant™ kit (cat. No. STRN50, SIGMA) from 40 to 45 mg of tissue.
288 RNA integrity was evaluated by 1% agarose gel electrophoresis, and its quality and
289 purity were determined using NanoDrop (ultradifferential spectrophotometer)
290 according to the 260/280 and 260/230 ratios. RNA concentration was quantified with
291 a Qubit™ RNA IQ assay (Invitrogen). The 18 sequencing libraries from poly(A)+
292 enriched RNA (Table S5) were prepared, and then sequenced in a Hi-Seq 4000 in a
293 150PE sequencing lane at the University of Berkeley, USA
294 (<https://www.berkeley.edu/>).

295 Demultiplexing was performed by the sequencing service. We performed
296 quality checks with FastQC and removed adapters and low-quality reads with

297 Trimmomatic (Bolger et al., 2014) using the following parameters: -phred33,
298 ILLUMINA CLIP: TruSeq3-PE-2.fa: 2: 30: 10, LEADING: 3, TRAILING: 3, SLIDING
299 WINDOW: 10 MINLEN: 50. Reads were mapped to the *Abies balsamea*
300 transcriptome (Van Ghelder et al., 2019; Bioproject PRJNA437248 in Genbank) with
301 BWA-MEM (Li & Durbin, 2009). Once the reads were mapped, we quantified the
302 transcript abundance by counting the mapped reads per transcript for each sample
303 (Table S6). Differential expression analyses were performed with DESeq2 (Love
304 et al., 2014) and edgeR (Robinson et al., 2010) in R for the following comparisons:
305 (1) symptomatic vs. asymptomatic individuals during the high O₃ concentration
306 period (170 ppb); (2) asymptomatic trees during the moderate (87ppb) vs. high O₃
307 concentration (170ppb) periods; and (3) symptomatic individuals during the
308 moderate (87ppb) vs. high O₃ concentration (170ppb) periods.

309 Transcripts with *p*-values lower than 0.005, after fold change correction
310 (Benjamini et al., 2001), were considered differentially expressed. Only those
311 transcripts detected by both methods were retained and analysed for identifying the
312 most likely open reading frames. They were then annotated with TRAPID 2.0 (Van
313 Bel et al., 2019) and BLASTx (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) using the
314 non-redundant database (nr); we retained the first five hits for each transcript. For
315 those transcripts that could not be annotated, we performed BLASTx searches
316 against theGymnosperm transcriptomes available at the Congenie
317 database(congenie.org). Proteins of annotated transcripts were finally assigned to
318 their respective metabolic pathways using KOALA (KEGG Orthology And Links
319 Annotation (Kanehisa et al., 2016)).

320

321 Results

Genotyping and geographic origin of trees

After *de novo* assembly and filtering, 1,550 SNPs were genotyped for the 88 retained *A. religiosa* individuals distributed along most of its range (Giles-Pérez et al., 2022), and for the ten focus samples of this study. Although the optimal number of genetic clusters (K) for the Admixture analysis was 2, a higher value ($K = 5$) had a better resolution for differentiating groups in the eastern and western most parts of the species distribution, allowing individual assignment. Both the symptomatic and asymptomatic trees of this study were assigned to the central-Mexico cluster, to which trees from neighboring populations, such as Ajusco and Nevado de Toluca also belong (Fig. 3). This result indicates that only local germplasm was included in our study.

Anatomical differentiation

Tissue differences were found between symptomatic and asymptomatic trees and among growth years (*i.e.*, needles developed in 2015 and 2016 and sampled in 2017) within individuals (Fig. 2b, Fig. S2). Needles of symptomatic trees exhibited a thicker epidermis and more collapsed cells than those of the asymptomatic ones, mainly within the palisade parenchyma (Fig. 2b). In contrast, the spongy parenchyma, resin channels and vascular tissues looked similar in the needles of symptomatic and asymptomatic individuals. Cell collapse became more evident with needle age in symptomatic trees (*i.e.*, higher for 2015 than for 2016 needles), while asymptomatic individuals showed less cell collapse in the two-year-old needles (2015) than in the one-year-old needles (2016; Fig. 2b).

Terpenes analysis

Compounds identified in all extracts included: δ -cadinene, α -cubebene, β -cubebene, α -caryophyllene, β -caryophyllene oxide, L- α -bornyl acetate, and β -pinene (Fig. 4). The best model for explaining the differences in concentration of these shared terpenes (Nagelkerke's $R^2 = 0.645$), indicated an association with the tree's condition (symptomatic and asymptomatic) and the period of exposition (87 ppb vs 170 ppb), with needle age being less relevant. Indeed, concentrations of all shared terpenes exhibited significant differences ($p < 0.001$, $p < 0.01$, or 0.05, Fig. 4) between symptomatic and asymptomatic individuals during the period of moderate ozone concentration. In addition, there were statistical differences in the terpene concentrations of asymptomatic trees between periods (87 ppb vs 170 ppb), but no differences were found between periods for the symptomatic trees or between needle ages (one- or two-years).

Differential expression analyses (RNA-seq)

After quality filtering, 605,147,387 paired reads were retained for 18 samples, with an average of 33,619,299 reads per sample. The percentage of reads mapped to the reference transcriptome (*A. balsamea*) ranged between 84.5 % and 96.7% per sample (Table S6), indicating excellent transcript coverage. Eleven differentially expressed transcripts were identified in the needles of the symptomatic and asymptomatic trees (fold change) during the high O_3 concentration period using both the DESeq2 and edgeR methods (Fig. 5a). Five of them were upregulated and six were downregulated in asymptomatic individuals. Six of these transcripts could be

368 annotated (Table S1) and were involved in carbohydrate metabolism, gene
369 regulation, and defense, according to KOALA. All of these transcripts belong to gene
370 families whose members are involved in different aspects of abiotic and biotic stress
371 response (see Table S1 for details), four of which have been previously associated
372 with O₃ response in controlled experiments with plants: *LRR receptor-like protein*
373 *kinases* (two annotated transcripts), an *L-type lectin-domain containing receptor*
374 *kinase*, and a *chitinase* (Table S1).

375 When comparing transcript expression between trees with the same
376 phenotype collected during low and high O₃ concentration periods, we observed six
377 and twenty-two differentially expressed transcripts for the symptomatic and
378 asymptomatic individuals, respectively; 17 of which could be annotated (Fig. 5b-c,
379 Table S2-3). Remarkably, the number of differentially expressed transcripts in the
380 asymptomatic plants was almost four times higher than that in symptomatic trees.

381 Among the five upregulated transcripts differentially expressed between
382 periods in the symptomatic individuals, two transcripts were involved in the
383 regulation of gene expression (encoding a *NAC* transcription factor and histone 1.3
384 variant) and one was involved in cell wall remodeling (encoding a *xyloglucan*
385 *endotransglucosylase*). The only downregulated transcript for these symptomatic
386 trees encoded an enzyme from the *UDP-glucosyl transferase* family involved in
387 various metabolic processes, including flavanol, tetrapyrrole, and terpene
388 biosynthesis (Table S2). Homologues in other plant species for four of the
389 upregulated transcripts have been previously associated with ozone response,
390 including the abovementioned *NAC* transcription factor and *UDP- glucosyl*
391 *transferase* (Table S2).

For the asymptomatic trees, 16 of the 22 differentially expressed transcripts between periods could be annotated (Table S3). For two of them, no homologous amino acid sequences were found, but the results of BLASTn performed in the Congenie database suggest that these could respectively represent a conifer specific non-coding RNA, and a conifer-specific peptide or protein. As for the annotated transcripts of these symptomatic individuals, they belong to gene families involved in response to abiotic and biotic stress, and the regulation of gene expression, four of these transcripts have been reported in controlled O₃ experiments in plants (Table S3). Interestingly, these include the *linker histone H1*, which was also upregulated in the symptomatic trees during the high O₃ concentration period.

Discussion

In this study, we explored the histological, metabolomic, and transcriptomic changes between symptomatic and asymptomatic fir trees within a natural population that has been heavily exposed to tropospheric O₃ for over 40 years. According to our genetic ancestry analysis, all the studied individuals belong to the local gene pool, which suggests that the observed differences are the likely result of intrinsic evolutionary processes within this population. Such differences include histological traits whose disparity increases with needle age, and contrasting terpene composition and gene expression. Our results illustrate how signals of O₃ tolerance can arise in a natural population after a few decades of frequent exposure and shed light on the metabolic and gene regulation mechanisms involved in conifers.

Asymptomatic trees have a local genetic origin

Comparing the genetic ancestry of our focus trees with other populations allowed us to confidently assign them to the previously reported central-Mexican genetic cluster (Giles-Pérez et al., 2022; Fig. 3b). This is important given that various reforestation efforts with foreign germplasm have been performed in the study zone and that some provenances have shown differential sensitivity to O₃ (Hernández-Tejeda & Benavides-Meza, 2015). Given that reforested trees have still not reached reproductive maturity, O₃ tolerance at the study site is the likely product of local processes, based on either plasticity or standing genetic variation (see below). Should genetic factors be involved, we hypothesize that only a relatively large effective population size could allow for the rapid evolutionary changes that are necessary to respond to such a strong environmental pressure in such a short term (1-2 generations if we consider a generation time of 25 years for sacred fir). Detailed quantitative and population genomics studies are thus necessary to evaluate tolerance heritability, estimate demographic parameters, and pinpoint the genomic bases of such putative adaptation.

Histological O₃ damage begins after only a few days of exposure

Overall, the symptoms observed herein were similar to those reported for other plant species experimentally exposed to O₃ under controlled conditions, at both the macroscopic and histological levels (Chaudhary & Rathore, 2021; Moura et al., 2022). Such symptoms are different from those expected from other possible stresses, such as drought or disease, which produce yellowish needles and a more homogeneously affected foliage (including needle loss; Chastagner, 2001; Johnson et al., 2005). In contrast, in this study, the reddish needle symptoms indicative of O₃

damage were first observed in 2-year-old needles, and foliage loss was limited to 3-year-old or older needles.

At the histological level, the needles of all individuals bore signs of damage, albeit to a much lower degree for the asymptomatic trees than for the symptomatic individuals (Fig. 2b, S2). This suggests a multivariate response to O₃ exposure that results in a continuous rather than in a discrete phenotype, likely controlled by polygenic or epigenetic factors. Our data further shows that O₃ damage begins at the tissue level during the first 30 days after bud burst (2017 buds; Fig. 2b), even if symptoms are still not noticeably macroscopically. Such precocious signs have been described for other conifers, for which they could appear as early as the fifth day of exposure (Evans & Fitzgerald, 1993). Both the visible and histological damages in firs aggravate with needle age (Fig. 2), which indicates a cumulative and irreversible effect of O₃ exposure (Schraudner et al., 1998), similar to that reported in controlled experiments in other plant species (Lee et al., 2020).

Cell collapse was particularly important within the palisade parenchyma (Fig. 2b, S2; (Alvarez et al., 1998; Evans & Fitzgerald, 1993; Terrazas & Bernal-Salazar, 2002), which has been attributed to oxidizing agents that act on the middle lamella of the cell wall and promote its degradation (Gimeno & Ibars, 2009). Such degradation increases intercellular spaces and leads to cell death (Alvarez et al., 1998), and it is often accompanied by the accumulation of phenolic and tannin compounds that produce the characteristic reddish coloration of O₃ damage (Fig. 2b, S2; (Gostin, 2010).

Symptomatic individuals had thicker epidermis than asymptomatic individuals (Ep; Fig. 2b). Such thickening has already been associated with O₃ response in conifers (Kivimäenpää et al., 2017) and might indicate increased synthesis of cell

465 wall components under O₃ stress (Sandermann et al., 1997). Interestingly, we did not
466 find any differences in cuticle and resin duct structure between symptomatic and
467 asymptomatic trees (Fig. 2b, S2), which was reported as a recurrent sign of O₃
468 damage in pines (Vollenweider et al., 2003). This suggests that either firs have a
469 greater tolerance to O₃ than pines or that such symptoms can only be observed
470 when comparing individuals unexposed and exposed to O₃ (which was impossible to
471 settle in our study, because there are no zero-exposure periods in our study site
472 throughout the year). Our own casual field observations suggest that pines (*i.e.*,
473 *Pinus ayacahuite*, *P. harwegii* and *P. veitchii*) growing in the study site seem to be
474 more affected than firs in terms of mortality, needle loss, and needle coloration.

475
476 *Asymptomatic trees produce terpenes related to response to biotic and abiotic stress*
477 *and recovery after stress*

478 Changes in cell structure in ozone-damaged plants may result from rampant
479 oxidative stress (Baier et al., 2005; Iriti & Faoro, 2008). These may be produced by a
480 deficient regulatory response, which results in the differential accumulation of certain
481 metabolites, including terpenes (Kopaczyk et al., 2020; Miyama et al., 2019).
482 Although we observed no clear anatomical differences in the resin ducts between
483 symptomatic and asymptomatic trees, which could have indicated contrasting
484 metabolite accumulation (Fig. 4), there were significant differences in terpene
485 composition, particularly sesquiterpenes, between asymptomatic and symptomatic
486 phenotypes during the moderate O₃ period. This is particularly compelling because
487 sesquiterpenes, which were also found to increase their concentration in
488 angiosperms when exposed to O₃ (Kanagendran et al., 2018; Pellegrini et al., 2012),

have been shown to degrade reactive oxygen species (ROS) and reduce cellular damage (Loreto & Fares, 2007; Vickers et al., 2009).

In our study, sesquiterpenes such as β -pinene, Δ -cadinene and β -caryophyllene were observed at higher concentrations in the asymptomatic than the asymptomatic trees prior to the high O₃ concentration period (Fig. 4). Such compounds have been associated with antioxidant and larvicidal functions in several plant species, including pines (Govindarajan et al., 2016; Kanagendran et al., 2018; Loreto et al., 2004; Ortiz de Elguea-Culebras et al., 2017). These terpenes could be allowing the asymptomatic trees to better cope with biotic and abiotic stresses once O₃ exposure increases (Pellegrini et al., 2012). The whole biosynthetic pathway leading to these compounds should be of particular interest for future functional and evolutionary studies in firs and other plants. However, given that insects often attack already weakened trees (like those exposed to O₃), such studies should also focus on disentangling the metabolic response to ozone exposure and insect defense.

Asymptomatic trees further produced a larger quantity of metabolites related to recovery after stress than symptomatic plants when we compared the metabolite composition between moderate and high O₃ periods (Fig. 4). Particularly β -pinene, which has been previously related to the plant recovery after a high O₃ exposure in *Nicotiana tabacum* (Kanagendran et al., 2018). This reinforces the idea that O₃ exposure is the main cause of forest degradation at our study site.

The members of the family of *UDP-glycosyltransferase* (UGT) enzymes participate in terpene biosynthesis (AB_008838_T.1; Table S2). The lower concentration of terpenes during the high O₃ period (Fig. 4) may be associated with the down-regulation of these transcripts in symptomatic trees when comparing the low (87 ppb) and high (170 ppm) O₃ concentration periods (Table S2). However, our

study should be complemented by examining the concentration of other metabolites, like flavonoids or tannins, in the future. Indeed, our results indicated that the expression of transcripts involved in the flavonoid metabolic pathway could exhibit considerable differences compared with those found for terpene metabolism, as demonstrated by the transcriptomic data (AB_000811_T.1; Table S1). In any case, the metabolic signatures reported here could already be used to identify trees that are not adequately recovering after O₃ exposure in affected forests.

Transcripts related to stomatal opening and response to stress are up-regulated in asymptomatic trees

To further examine the molecular basis of O₃ response, we performed a differential transcript expression analysis (DTE). We found differentially expressed transcripts when comparing asymptomatic and symptomatic trees during the high O₃ concentration period (Table S1, Fig. 5a) and when independently comparing concentration periods for individuals with the same phenotype (Table S2-S3, Fig. 5b-c). Homologs of several of these transcripts have been previously reported as differentially expressed in controlled O₃ exposure experiments in angiosperms (Natali et al., 2018; Tammam et al., 2019; Waldeck et al., 2017), which suggests that the molecular mechanisms underlying response to O₃ are conserved on a large evolutionary time scale.

The differentially expressed transcripts during high O₃ concentration periods were associated with defense against pathogens and stomata opening, and included transcripts related to chitinases and LRR protein kinases. These proteins are known to play important roles in recognizing and responding to pathogens in plants (Vaghela et al., 2022; Wang et al., 2023), and their differential expression suggests

either a response to an unaccounted pathogen attack (e.g., fungi) or that this signaling pathway is activated under both O₃ exposure and other stressors. Again, this indicates the need for further studies to disentangling the response to O₃ and biotic stress defense. Interestingly, some members of the LRR kinases gene family are also associated with the initial physiological reaction of plants to O₃ exposure, which involves stomatal closure (Hasan et al., 2021). Thus, studying stomata closure, and its underlying genes, should be a priority for future studies in natural plant populations affected by O₃ pollution.

Comparing transcriptional profiles among trees with the same phenotype, asymptomatic or symptomatic, also showed differential responses to increased O₃ concentration. In other words, the upregulated and downregulated transcripts belong to different GO categories. Among the upregulated transcripts in symptomatic individuals during the moderate O₃ period (Fig. 5b, Table S2), a homolog of the *xyloglucan endo-transglycosylase* and a *non-apical meristem* (NAM) transcription factor from the large NAC family stand out, as some of their homologs have been shown to play a key role in cell repair after O₃ exposure (Zhang et al., 2017) and are activated by O₃ during apoplastic ROS signaling (De Clercq et al., 2013). The activation of these pathways in symptomatic trees when O₃ concentration is low, might be indicative of decreased sensitivity to this pollutant when compared to the asymptomatic trees.

During the high O₃ period, asymptomatic individuals upregulated some transcripts (Fig. 5c, Table S3) related to plant resistance (NB-ARC-domain proteins), plant defense (peroxidases), and the flavonoid biosynthesis (chalcones) pathway (Dao et al., 2011; Krasensky et al., 2017). In other words, when O₃ concentration increases, asymptomatic trees may be activating mechanisms related to stress

response. Moreover, transcripts encoding for *UDP-glycosyltransferase (UGT)* family members (Fig. 5b, Table S2), which are essential components of the plant secondary metabolism pathway that helps detoxify harmful compounds (Pan et al., 2019), are downregulated in asymptomatic trees. *UGTs* are also essential for regulating various aspects of plant growth and development (Mateo-Bonmatí et al., 2021).

All in all, the variety of pathways differentially activated between symptomatic and asymptomatic trees highlights the complexity of studying plant transcriptomic responses in natural conditions (Nunn et al., 2006). Indeed, several sources of stress are expected to act at the same time in degraded forests subjected to air pollution. To disentangle the various mechanisms involved, it is advisable to use controlled experiments, such as ozone top chambers (Abeyratne & Ileperuma, 2006; Palomäki et al., 1998), in combination with *in situ* studies in natural settings to understand how plants respond to stress under real-life scenarios. However, although several sources of stress are at play in peri-urban forests of Mexico City, our histological, terpenes, and transcriptomic analyses confirm that O₃ pollution is an important stressor that triggers a rapid and differential phenotypic response in firs, likely modeled by standing genetic variation and/or plastic mechanisms. The evolutionary basis of such differences remains open to be explored. Since epigenetic variation is related to gene activity and expression (Richards et al., 2017; Srikant & Drost, 2021), and can accumulate faster than DNA mutations, their role in the phenotypic response to O₃ pollution must be addressed in future studies.

Data accessibility and benefit-sharing

Histological images and processed terpenes, genotype (vcf files) and transcriptomic (expression tables) data are available at the Dryad repository XXXXX (available upon acceptance). Pipelines and code for all analyses is available at the Github repository (https://github.com/Verolarrachtai/Abies_religiosa_vs_ozone). Transcriptome raw sequences data were deposited in GeneBank under accession numbers XXXX (available upon acceptance). Demultiplexed sequencing data, including those samples previously analyzed in a phylogenetic survey (i.e., 80 samples, Giles et al.,2022), were deposited in NCBI with the Bioproject ID: PRJNA856692; while filtered variant files used for population genomic analyses, code and pipelines are hosted on Dryad Repository at XXXX (available upon acceptance) and on GitHub at XXXX (available upon acceptance).

Author contributions

VRG, CZC and AMY performed sampling. VRG performed lab work and analyses. VRG, JPJC and AMY designed the study, interpreted results, and drafted the manuscript. LS, CAM, SS, ESZ, RTJ, CMF and DP contributed to data analyses and interpretation. All authors produced and approved the final version of the manuscript.

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621

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