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Impacts of greenhouse gases on epicuticular waxes of *Populus tremuloides* Michx.: Results from an open-air exposure and a natural O_3 gradient

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Structure of epicuticular waxes indicated phytotoxic effects of greenhouse gases on Populus tremuloides Michx.

Abstract

Epicuticular waxes of three trembling aspen (*Populus tremuloides* Michx.) clones differing in O_3 tolerance were examined over six growing seasons (1998–2003) at three bioindicator sites in the Lake States region of the USA and at FACTS II (Aspen FACE) site in Rhinelander, WI. Differences in epicuticular wax structure were determined by scanning electron microscopy and quantified by a coefficient of occlusion. Statistically significant increases in stomatal occlusion occurred for the three O_3 bioindicator sites, with the higher O_3 sites having the most affected stomata for all three clones as well as for all treatments including elevated CO_2, elevated O_3, and elevated CO_2+O_3. We recorded statistically significant differences between aspen clones and between sampling period (spring, summer, fall). We found no statistically significant differences between treatments or aspen clones in stomatal frequency.

Keywords: *Populus tremuloides* Michx; O_3 tolerance; Epicuticular wax; Stomatal frequency

1. Introduction

Global atmospheric and pre-industrial CO_2 concentrations are expected to double by the end of the next century (Keeling et al., 1995). Troposphere ozone (O_3), a secondary pollutant generated from nitrogen oxides (NO_x) and volatile organic compounds (VOC) from fossil fuel, such as thermal generation and transportation is also increasing globally. At the same time, forest tree species are exposed to the effect of CO_2, O_3 and other pollutants. While CO_2 generally stimulates tree growth and O_3 and other air pollutants generally decrease tree growth, there is little information available on the impacts of interaction of CO_2 and O_3 on epicuticular waxes and stomatal frequency. Trembling aspen is a good model species to examine the effects of these two pollutants, as it is highly responsive to both CO_2 and O_3. Furthermore, we have identified a wealth of genetic variation in the response of trembling aspen to air pollutants, and we have isolated O_3-sensitive and tolerant clones (Dickson et al., 2000; Karnosky et al., 1998, 1999).

Elevated CO_2 and O_3 affect trees through different mechanisms. With trembling aspen elevated CO_2 stimulates photosynthesis (Tjoelker et al., 1998), delays foliar senescence in autumn and stimulates aboveground

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(Isebrands et al., 2001) and belowground (King et al., 2001) growth. Trees grown with elevated CO_2 generally have lower nitrogen concentrations in their foliage, lower Rubisco concentrations, altered defense compounds and decreased concentration of antioxidants (Lindroth et al., 1993, 1997).

In contrast to the largely beneficial effects of CO_2 on aspen, O_3 is generally detrimental to aspen growth and productivity. Ozone has been shown to induce foliar injury (Karnosky, 1976), decrease foliar chlorophyll content, accelerate leaf senescence (Karnosky et al., 1996), decrease photosynthesis (Coleman et al., 1995a), alter carbon allocation (Coleman et al., 1995b, 1996), alter epicuticular wax structure and composition

Table 1
Classification of changes of the epistomatal wax of *P. tremuloides*

Class 1	A maximum of 10% of the total stomatal area show the beginnings of fusion of single wax tubules
Class 2	Several of the apically aggregated wax tubules fuse to small wax tufts at different parts of the epistomatal area. The latter cover 10–25% of the total stomata area
Class 3	In addition to the wax tufts plate-like wax parts can be found that, in total, cover more than 25% and up to 50% of the total stomata area
Class 4	More than 50% and up to 75% of the total stomata area show small parts of wax tufts as well as large platelet wax forms
Class 5	More than 75% of the total stomata area is characterized by considerably changed wax microstructures. The stomata antechamber is almost or completely occluded with an amorphous wax plug

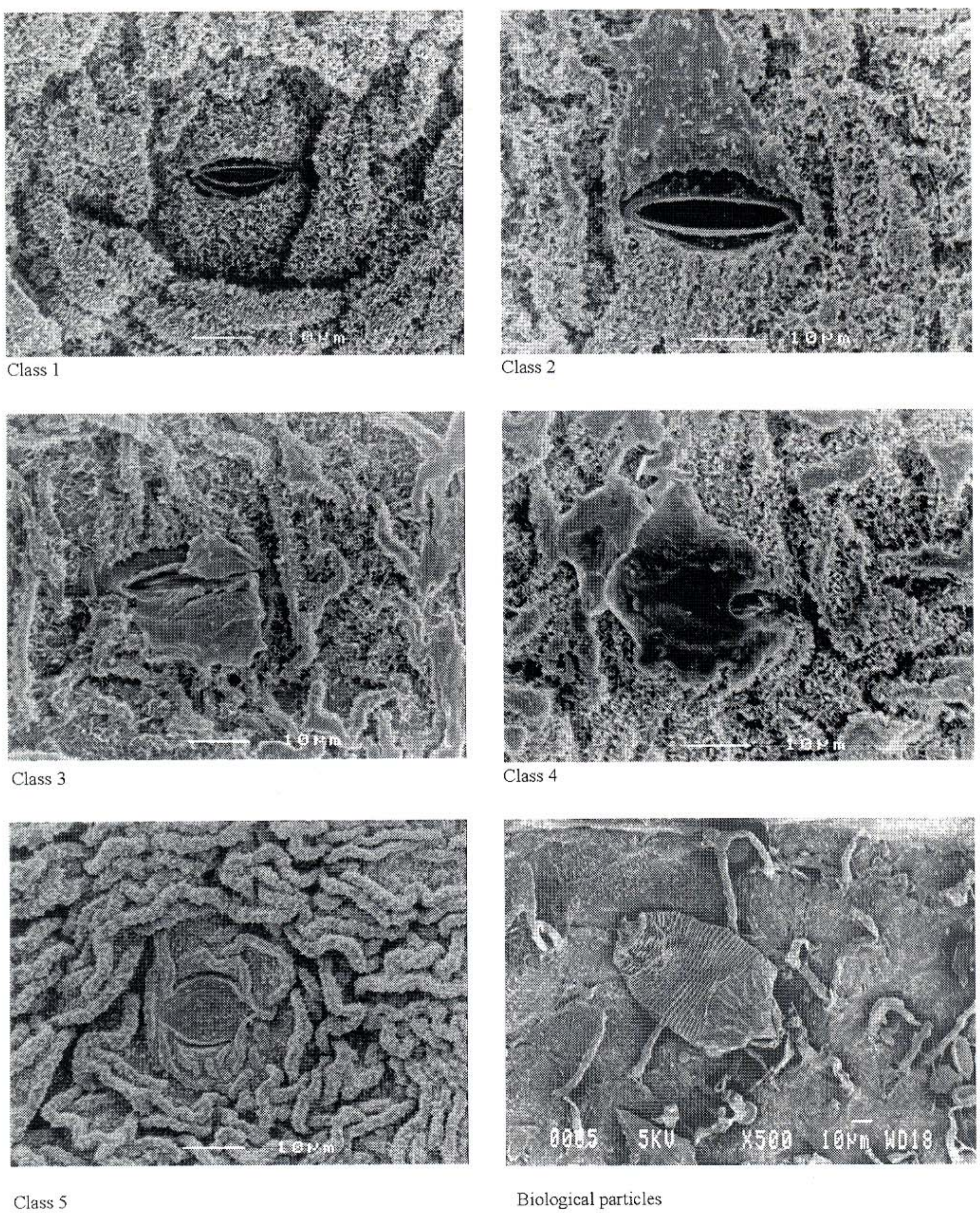

Fig. 1. Micrographs of trembling aspen stomata.

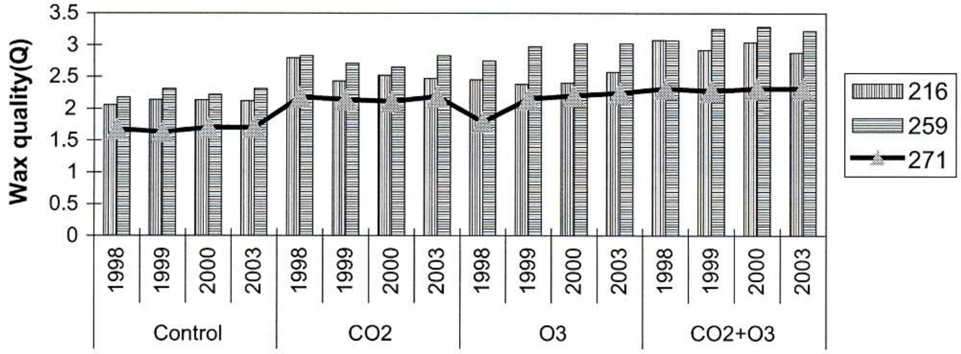

Fig. 2. Wax quality (Q) of clones 216, 259 and 271 (median) of *P. tremuloides* between 1998 and 2003 at Aspen FACE.

(Maňkovská et al., 1998; Karnosky et al., 1999), and decrease growth (Wang et al., 1986; Karnosky et al., 1992; 1996, 1998; Coleman et al., 1996).

The overall objective of the work is to compare: (a) the impacts of elevated levels of O_3, and CO_2 on epicuticular wax formation; (b) the seasonal development of stomatal frequency; (c) the impact of a $1.5\times O_3$ treatment in an open-air exposure system to that in a naturally high ambient O_3 area downwind of a major metropolitan area. In addition we examined the effects of elevated O_3, and CO_2 on stomatal frequency and in rust colonization on aspen leaf surfaces.

2. Materials and methods

Foliage of three trembling aspen (*Populus tremuloides* Michx.) clones differing in O_3 tolerance (clone 259, O_3 sensitive; clone 216, O_3 intermediate; and clone 271, O_3 tolerant) were examined during six growing seasons (1998–2003). Fully-expanded leaves (LPI 5 to 8) were sampled from the south side of the upper crown of each tree. Leaves were generally sampled in August of each year except in years when we sampled early in each month of June, July, and August. Stomatal frequency was examined in 2000. Rust frequency was examined in 2003. The clones were growing at three locations in a "natural" O_3 gradient from Rhinelander, northern Wisconsin (low O_3, 1996 SUM00 = 41.0 ppmh), to Kenosha, southern Wisconsin (high O_3, 1996 SUM00 = 70.4 ppmh) to Kalamazoo, southern Michigan (intermediate O_3, 1996 SUM00 = 47.3 ppmh) (Karnosky et al., 1999, 2002, 2003). The Rhinelander locality encompassed 32 ha of land with 12 30-m diameter treatment rings spaced 100 m apart within a deer-fenced area. The rings were composed of three control rings, three rings with elevated O_3, three rings with elevated CO_2, and three rings with elevated O_3+CO_2 (Dickson et al., 1998).

Air-dried leaves were treated by JEOL Ion sputtering prior to observation. They were assessed by scanning microscope JEOL 840 A. The wax surface SEM was done at the Canadian Forest Service's Fredericton, New Brunswick and at the Forest Research Institute, Zvolen. The wax quality was determined by evaluation of 200 stomata per leave. Two leaves were evaluated per clone and month per site. Quantification changes in the epistomatal wax structure of five distinct classes were defined by two criteria: different crystal wax morphology and the varying degree of changed wax structures to

Table 2
Analysis of variance for changes of epistomatal waxes of *P. tremuloides* (three clones) at Aspen FACE (four treatments) between 1998 and 2003

	Degrees of freedom	Variance	*F*-test	Level of significance	Significance
Treatment	3	0.6830	74.88	99.99	***
Years	1	0.0010	0.08	22.50	N
Clones	2	1.1490	125.91	99.99	***
Treatment × years	3	0.0330	3.57	91.35	N
Treatment × clones	6	0.0230	2.46	85.21	N
Years × clones	2	0.0310	3.36	89.53	N
Residual	6	0.0090			
Together	23	0.2000			

Table 3
Variance analysis for changes of epistomatal waxes in *P. tremuloides* (three clones) in Rhinelander, Kenosha and Kalamazoo between sampling periods

	Degrees of freedom	Variance	*F*-test	Level of significance	Significance
Localities	2	1.4040	236.60	99.99	***
Clones	2	1.9970	336.56	100.0	***
Month	2	0.1850	31.13	99.98	***
Localities × clones	4	0.1680	28.38	99.99	***
Localities × month	4	0.0330	5.47	97.98	*
Clones × clones	4	0.030	5.37	97.88	*
Residual	8	0.0060			
Together	26	0.3100			

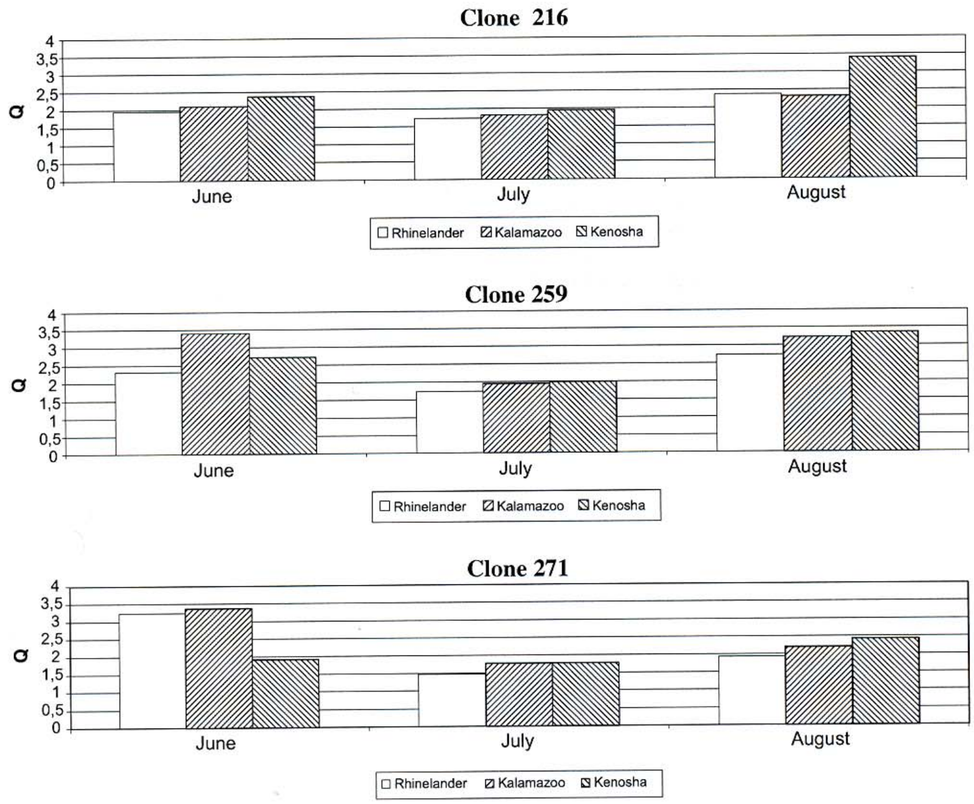

Fig. 3. Wax quality (Q) of three clones of *P. tremuloides* between different sampling period and localities (Rhinelander, Kalamazoo and Kenosha).

the stomata area (Maňkovská, 1996; Maňkovská et al., 1998, 2003a,b; Trimbacher and Eckmüllner, 1997) (Table 1). We used C_o, the coefficient of occlusion (arithmetical mean of wax quality of 200 stomata per leaf) (Fig. 1). The vegetation samples were evaluated by statistical test ANOVA.

3. Results and discussion

The worst wax quality was found for the most sensitive clone 259 and for the treatment with O_3+CO_2. Quality of waxes for clone 216 was better than for clone 259, except for the treatment O_3, where WQ in all observed years was higher than WQ for clone 259. The best quality of waxes was found for clone 271, whereas the highest value of WQ was found for the treatment O_3+CO_2 (Fig. 2). We found statistically significant differences in quality of stomata between three aspen clones and four treatments in the Rhinelander locality when comparing results of quality of stomata. Statistically significant differences were found between years, between years and treatments, between treatments and clones, and between years and clones (Table 2).

When comparing results we have found the largest differences in wax quality of stoma between individual

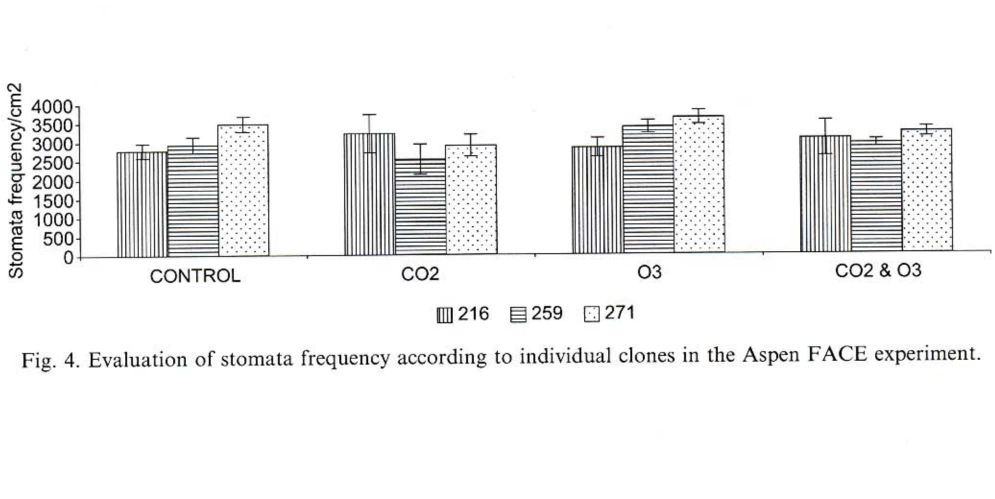

Fig. 4. Evaluation of stomata frequency according to individual clones in the Aspen FACE experiment.

Table 4
Evaluation of stomata frequency according to individual clones in the Aspen FACE experiment

Treatment	No. of stomata (cm^2) (SD)		
Clone	216	259	271
Rhinelander			
Control	2779 (192)	3613 (1472)	4599 (1437)
CO_2	3216 (509)	2245 (1201)	6953 (579)
O_3	2833 (250)	5201 (710)	8390 (616)
O_3	3066 (475)	6377 (1180)	5748 (226)
Kenosha	3695	1027	6200
Statistical evaluation			
Treatment[a]	Insignificant		
Clones	Insignificant		
Treatment × clones	Insignificant		
Locality[b]	Insignificant		
Clones	Insignificant		
Locality × clones	Insignificant		

Values in the table are means (standard error of mean). ANOVA, $*P<0.05$, $**P<0.01$, $***P<0.001$. Means designated with different letters are different ($P<0.05$, Turkey's multiple range test).

[a] Results of analysis of variance for Rhinelander; we tested the influence of treatments O_3, CO_2, O_3+CO_2 and clones 216, 259, 271 on the number of stomata.

[b] Results of analysis of variance; we tested the influence of localities Rhinelander (we calculated only with variant control, Kenosha and clones 216, 259, 271 on number of stomata.

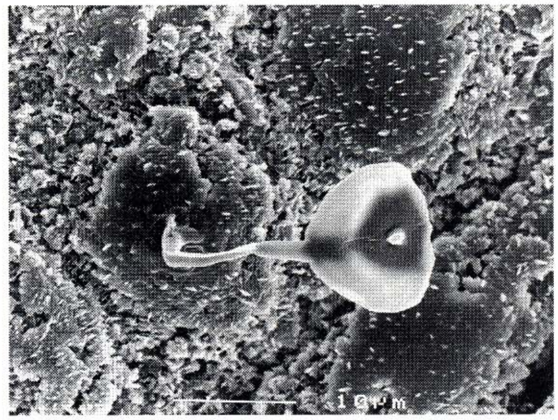

Fig. 6. Aspen surface with spores.

aspen clones at the Kenosha locality. Tolerant clone 271, unlike 216 and 259 had most relatively undamaged stoma in class 1 and 2. Clone 259 had most damaged stoma in class 4 and 5. When comparing wax quality of stoma from Kenosha with locality Rhinelander there has been found statistically significant difference between individual aspen clones with the exception of clone 216 (CO_2+O_3) (Table 3). Arithmetical mean of wax quality for individual clones of aspen for Rhinelander–Kenosha–Kalamazoo in June, July, and August in 1999 are shown in Fig. 3. We found statistically significant differences in wax quality of stomata between three localities, three aspen clones, three months and four treatments at the Aspen FACE (control, $+CO_2$, $+O_3$, $+CO_2+O_3$) in the localities when comparing results of wax quality of stomata (Table 3).

We did not find any statistically significant differences in the frequency of stomata (Fig. 4, Table 4).

We found statistically significant differences in rust colonization on aspen leaf surfaces at the Aspen FACE site (Figs. 5 and 6, Table 5).

Statistically significant increases in stomatal occlusion occurred for three O_3 bioindicator sites as we predicted with the higher O_3 sites having the most affected stomata for all three clones as well as for all treatments including elevated CO_2, elevated O_3, and elevated CO_2+O_3. Our results suggest that O_3 pollution of the Kenosha and Kalamazoo sites shows significant negative impact on wax quality of aspen and this impact is the most severe on the most O_3 sensitive clones. We recorded statistically significant differences between aspen clones, between sampling period (spring, summer, autumn) and localities between Rhinelander, Kalamazoo and Kenosha. However, we found no statistically significant differences between treatments or aspen clones in stomatal frequency. We monitored stomatal frequency of aspen clones only in 2000. In our next work, it will be necessary to monitor these changes every year. We think that stomatal frequency is genetically given for individual observed aspen clones in Rhinelander. Similarly it will be necessary to monitor differences in rust colonization on aspen leaf surfaces every year.

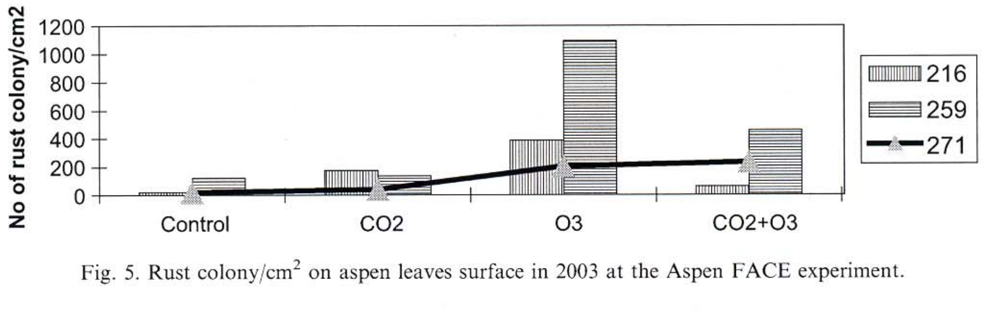

Fig. 5. Rust colony/cm^2 on aspen leaves surface in 2003 at the Aspen FACE experiment.

Table 5
Statistical evaluation of rust colony/cm^2 on aspen surface in 2003

Treatments	Clones	Control		CO_2			O_3			CO_2+O_3		
		259	271	216	259	271	216	259	271	216	259	271
Control	216	-0.14^{N}	0.44^{*}	-0.44^{*}	-0.64^{*}	-0.04^{N}	-0.34^{N}	-0.83^{*}	0.018^{N}	-0.87^{*}	-1.09^{*}	-0.19^{N}
	259	–	0.58^{*}	-0.30^{N}	-0.50^{*}	0.10^{N}	-0.20^{N}	-0.69^{*}	0.158^{*}	-0.73^{*}	-0.96^{*}	-0.05^{N}
	271	–	–	-0.88^{*}	-1.08^{*}	-0.48^{*}	-0.77^{*}	-1.26^{*}	-0.42^{*}	-1.30^{*}	-1.53^{*}	-0.62^{*}
CO_2	216	–	–	–	-0.2^{N}	0.4^{*}	0.11^{N}	-0.39^{*}	0.46^{*}	-0.43^{*}	-0.65^{*}	0.26^{N}
	259	–	–	–	–	0.6^{*}	0.31^{N}	-0.19^{N}	0.66^{*}	-0.23^{N}	-0.45^{*}	0.455
	271	–	–	–	–	–	-0.30^{N}	-0.79^{*}	0.06^{N}	-0.83^{*}	-1.05^{*}	-0.15^{N}
O_3	216	–	–	–	–	–	–	-0.49^{*}	0.35^{*}	-0.53^{*}	-0.76^{*}	0.15^{N}
	259	–	–	–	–	–	–	–	0.85^{*}	-0.04^{N}	-0.27^{N}	0.64^{*}
	271	–	–	–	–	–	–	–	–	-0.89^{*}	-1.11^{*}	-0.21^{N}
CO_2+O_3	216	–	–	–	–	–	–	–	–	–	-0.23^{N}	0.68^{*}
	259	–	–	–	–	–	–	–	–	–	–	0.91^{*}

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