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SEARCH FOR RYE AND WHEAT GENOTYPES WHICH ARE RESISTANT TO *Claviceps purpurea* (Fr.) Tul. AND HAMPER ACCUMULATION OF ERGOALKALOIDS IN SCLEROTIA

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Abstract

Ergot [*Claviceps purpurea* (Fr.) Tul.] is a progressive disease of rye and wheat crops. Ergot alkaloids (EA) derived from the fungus can cause severe health problems in both humans and animals. Ergot-resistant cereals are the guarantors to solve the problem. Here, we aimed to determine the EA profiles and content in sclerotia of the Kirov population of *C. purpurea* and to search for rye and wheat genotypes resistant to ergot and with no EA accumulation to be used as sources of these traits in breeding. One hundred varieties of winter rye (*Secale cereale* L.) and spring soft wheat (*Triticum aestivum* L.) obtained from the collection of the Vavilov All-Russian Institute of Plant Genetic Resources (VIR) and bred at the Rudnitsky Federal Agricultural Research Center of the North-East were tested in 2017-2019 for ergot resistance by artificial inoculation of flowers with a suspension of *C. purpurea* conidia (3×10^6/ml). EA profiles and amount were estimated by thin layer chromatography on silica gel plates (Silica gel F_{254}, Merck, Germany). The metabolites were identified by co-chromatography with standards and by UV spectra (a UV-160A spectrophotometer, Shimadzu, Japan) and mass spectra (an LCQ Advantage MAX spectrometer, Thermo Finnigan, Germany). Most of the tested samples were susceptible to ergot. In wheat, only two varieties were immune (Novosibirskaya 18 and a new line T-66) and 13 varieties were relatively resistant with a lesion level of no more than 5.2 % and a sclerotia content in grain of no more than 0.3 % vs. 21.7 % and 1.5 % in the most susceptible (indicator) variety. In winter rye, immune forms were absent, in 10 varieties, the lesion varied from 5.8 % to 33.0 % and sclerotia contamination of grain from 0.3 % to 1.4 % vs. 100 % and 37.0 % in the indicator) variety). The immune and least affected varieties of rye and wheat can be used in breeding programs as genetic sources for the trait. The EA composition and amount in the fungus *C. purpurea* sclerotia were analyzed in 30 varieties of rye and wheat with different susceptibility to ergot. The EA composition was the same and consisted of ergocristine, ergotamine, and its stereoisomer ergotaminine. Only in the Kazakh wheat variety Samgau, we identified one EA, the ergotamine. The amount of EA in sclerotia varied from 0 % to 0.36 % of their weight for rye plants and from 0 to 2.40 % for wheat plants. Nine new rye populations bred at the at the Rudnitsky Federal Agricultural Research Center of the North-East and four wheat varieties do not accumulate EA. A weak negative relationship was found between the weight of a sclerotium and the EA accumulation, $r = -0.46$ (p = 0.05) for winter rye and $r = -0.32$ (p = 0.05) for spring wheat. The revealed tendency increases the biological hazard of the small and most difficult to separate fraction of sclerotia in the grain mass. No significant relationship was found between the toxicity and pathogenicity of the fungus *C. purpurea* as evidenced by the correlation between the ergot occurrence and the EA content in the rye ($r = 0.22$, p = 0.05). Nevertheless, data on the EA level are important for the search for immunologically and breeding-valuable genotypes that combine resistance to ergot and no EA accumulation. This trait is characteristic of the German wheat variety Epos and the new rye populations Rumba, Harmony, and Symphony.

Keywords: winter rye, spring wheat, ergot, *Claviceps purpurea*, sclerotia, resistant cultivars, ergo alkaloids, toxicity, pathogenicity

In cereals, ergot epidemiological situation caused by the fungus *Claviceps purpurea* (Fr.) Tul. (*Ascomycetes*), is recently worsening in many regions of the Russian Federation [1, 2] and abroad, especially in the Baltic republics, western Belarus, and Central Europe [3-5]. In Germany, an increase in *C. purpurea* infestation is due to larger crop area of high-yielding but more susceptible hybrid rye [6]. In Belarus, sclerotia in grain fodder consignments is of particular concern, since they are identical to rye caryopsis in shape and size which reduces the efficiency of their removal from the grain mass to 23% [7].

The disease expansion and increased severity are due to a number of reasons. A certain danger is posed by unused agricultural lands in which infectious elements of *C. purpurea* persist and accumulate. Many questions remain about modern plant growing technologies aimed at minimizing soil cultivation and other treatments. Intensifying climate change, together anthropogenic and technogenic factors, negatively impact field biocenoses. In the Kirov region, *C. purpurea* annually infects half of the sown winter grain crops. In rye biocenoses, the diseases affects 0.2-5.0% plants [8, 9]. Existing grain production technologies do not provide complete protection against ergot and the ingestion of sclerotia in seeds.

The tightening of Russian state standards (GOSTs) and foreign regulations aimes to prevent sclerotia contamination of reproduced high-quality original seeds [4, 6, 10] and to ensure the quality and safety of products. Consumption of grain and feed contaminated with sclerotia can cause poisoning for humans and animals [11-14]. Canadian researchers [15] detected up to six types of ergot alkaloids (EA) in the flour from batches of wheat grain with an extremely low (0.004%) amount of sclerotia. As per other report [16], ergot alkaloids are very stable when preparing various food products from such flour. The *C. purpurea* ability to produce EAs is genetically determined but the EA profiles and amounts depend on the host plant, geographic region and climatic factors [6, 17-20]. The most common EAs are ergometrine, ergotamine, ergocornine, ergocriptine, ergosine, and ergocristine (21, 22).

The Tatar Research Institute of Agriculture (Federal Research Center of the KSC RAS, Kazan) and the Federal Research Center Nemchinovka (Moscow Region) have focused their research on genetic protection against ergot. The gene pool of the main food crops — winter rye and spring wheat has practically not been studied for ergot resistance, especially under artificial inoculation. The EAs composition and content in the sclerotia of various grain crops and the mechanisms of plant resistance to *C. purpurea* are still poorly understood, which partly limits the progress in breeding for ergot resistance.

Genetic studies in the *Secale sereale* L.—*C. purpurea* system are difficult due to special biological traits of the pathogen and a complex procedure for the infection loading necessary to generate resistance donors. The lack of information on the phenotypic and genetic structure of populations of the genus *Claviceps* and biochemical markers of resistance are additional limitations. An important biomarker actively used in Germany is increased pollen production and pollen fertility [23]. Due to the significant influence of the environment on the pathogenesis in the *S. sereale*—*C. purpurea*, researchers note that reliable assessment of the resistance to ergot necessitates repeated testing of a genotype [23].

The total amount of EAs in sclerotia (toxicity) was shown to weakly and positively correlate with the *C. purpurea* pathogenicity to 100 new varieties of rye and wheat. Lab and field tests detected effective genetic sources of a combined plant resistance to ergot and EA accumulation in grain for use in breeding.

The work aimed to determine the total content and composition of ergot alkaloids in the *Claviceps purpurea* sclerotia collected from winter rye and spring bread wheat plants in the Kirov region, and to identify genotypes resistant to ergot and EA accumulation.

Materials and methods. Field phytopathological testing was carried out in 2017-2019 (the Rudnitsky Federal Agrarian Scientific Center of the North-East — FASC of the North-East). The genotypes tested were 26 new diploid (×2) varieties of winter rye (*Secale cereale* L.), 20 varieties of spring wheat (*Triticum aestivum* L.) (bred at the FASC of the North-East), 34 winter rye samples and 20 spring wheat samples (accessions from the VIR world collection, Federal Research Center Vavilov All-Russian Institute of Plant Genetic Resources) [24]. The gene pool of these cereals was investigated by plant inoculation with an aqueous suspension of *C. purpurea* conidia. The conidia were isolated from sclerotia freshly collected in the Kirov region on rye and wheat plants and stored on potato-glucose agar in the working collection (FASC of the North-East). The inoculated plants were compared to control plants not inoculated with *C. purpurea*.

Immediately before inoculation, the spores were washed off the surface of a pure culture of the pathogen with distilled water. The spore concentration was adjusted to 3×10^6 conidia/ml using a Goryaev chamber [25]. The inoculum was introduced into the ovary with a syringe in the period from heading to the beginning of flowering (phases 55-61 on the Zadoks scale). Ten to fifteen plants were inoculated in 3-fold repetition. Two indicators used to characterize the varieties were the percentage of the diseased plants (prevalence) and grain contamination as the amount of sclerotia per grain sample (w%). After threshing the inoculated plants, all sclerotia were separated from the grain, weighed, and their mass ratio to the grain mass was calculated. The collected sclerotia were described biometrically by weight and size and analyzed for EA qualitative and quantitative composition. Ergot resistance of cultivars was assessed according to the scale of Miedaner et al. [26]. The disease prevalence of up to 0.5% with no more than 0.01% sclerotia correspond to high resistance, up to 1.5% and up to 0.10% — to moderate resistance, and prevalence of more than 3.0% and up to 0.3% of sclerotia per grain weight means susceptibility of the genotype.

EA profiling was performed in 30 varieties of rye and wheat, different in susceptibility to ergot (Federal Research Center PSCBR RAS, Skryabin Institute of Biochemistry and Physiology of Microorganisms RAS, Pushchino). EA was extracted from 1 g of crushed sclerotia in two ways. In the first experiment, the samples were extracted three times with a 50% aqueous solution of acetone containing H_2SO_4 to create acidic conditions (pH 4.5). The bulk extract was concentrated on an IR-1M2 rotary evaporator (Khimlaborpribor, Russia) to half the initial volume. The resulting aqueous fraction was added with 25% ammonia solution to pH 9-10 and extracted 3 times with chloroform. Chloroform extracts were dried with anhydrous Na_2SO_4 and evaporated on a rotary evaporator. The second method consisted in the extraction of EAs with a mixture of chloroform and methanol (1:1).

The extracts were analyzed by thin layer chromatography (TLC) on silica gel plates (Silica gel F_{254}, Merck, Germany) in chloroform:methanol:25% NH_4OH solvent system, at 90:10:0.1 (I) and 80:20:0.2 (II). EAs were detected by absorption or fluorescence in UV light (λ = 264 nm) and after spraying the plates with Ehrlich's reagent. EAs were isolated and purified by preparative TLC on silica gel plates. Metabolites were identified by chromatography with standard samples and by UV spectroscopy (a UV-160A spectrophotometer, Shimadzu, Japan) and mass spectrometry (an LCQ Advantage MAX quadrupole mass spectrometer, Thermo Finnigan, Germany; direct sample injection into a chemical ionization chamber by a single-channel syringe pump at atmospheric pressure). The total amount of EAs in the extracts was determined spectrophotometrically in methanol at λ = 313 nm. The calculation was carried out using the molar extinction coefficient of ergotamine (log ε = 3.86). For each sample of the extract, all measurements were repeated at least 5 times.

Data were processed statistically using the methods of variance and correlation analysis. The calculation matrix included data on the disease prevalence and grain contamination with sclerotia. The significance of differences vs. the standard varieties of rye (Falenskaya 4) and wheat (Bazhenka) was estimated. The software package for statistical, biometric and genetic analysis AGROS (version 2.07) and Microsoft Office Excel were used. The EA concentrations are presented as the arithmetic mean (*M*). Confidence intervals for the EA levels (±SEM) did not exceed ±5% ($p \le 0.05$).

Results. The rye and wheat cultivars in the control presented no symptoms of the disease or had single sclerotia. With artificial inoculation, the cultivars ranged from immune to susceptible forms. In new diploid (×2) rye populations, the prevalence varied from 14.2 (cv. Leda) to 78.5% (cv. Niobe), in diploid accessions from the VIR collection — from 5.8 (Podarok NP) to 100% (cv. Benyakonskaya 2, cv. Getera 2); grain infestation with sclerotia varied, respectively, from 0.6 (Leda) to 7.6% (cv. Gratsia) and from 0.3 (cv. Podarok NP) to 37.0% (cv. Kompus).

1. Winter rye (*Secale cereale* L.) and spring soft wheat (*Triticum aestivum* L.) varieties resistant to *Claviceps purpurea* (Fr.) Tul. (*M*±SEM; artificial inoculation, field tests, Kirov, 2017-2019)

Variety. line, geographic origin	Ergot prevalence, %	Grain contamination with sclerotia, %
Winter rye		
Varieties from the VIR collection		
Podarok NP, Leningrad Province	5.8±0.50*	0.3±0.02*
Chulpan 2, Leningrad Province	14.2±2.80*	0.6±0.08*
Vavilovskaya NP, Leningrad Province	15.3±2.90*	1.0±0.09*
Rossiyanka 2, Leningrad Province	17.6±3.10*	0.9±0.25*
Triodis 4 — Minvak-139/09 NP, Leningrad Province	16.6±2.80*	0.6±0.09*
Krasnoyarskaya universal'naya NP, Leningrad Province	18.7±3.90*	0.8±0.19*
Average	46.6	4.9
Varieties bred at the Rudnitskii FASC of the North-East		
Leda	14.2±2.80*	0.6±0.08*
Simfoniya	19.5±4.20*	1.2±0.28*
Garmoniya	30.7±5.70*	1.2±0.29*
Rumba	33.0±6.40*	1.4±0.31*
Falenskaya 4 (standard)	40.7±8.20	3.5±0.75
Average	57.4	3.6
Benyakonskaya 2 (reference variety)	100	20.0±6.80
Kompus (reference variety)	50.0±8.80	37.0±9.90
Spring soft wheat		
Varieties from the VIR collection		
Novosibirskaya 18, Novosibirsk Province	0*	0*
Tulaikocskaya nadezhda, Samara Province	1.3±0.11*	0.1±0.01*
Kayir, Kazakhstan	1.3±0.11*	0.1±0.01*
Ul Alta Blanca, USA	1.6±0.18*	0.1±0.01*
Epos, Germany	2.1±0.52*	0.2±0.03*
Samgau, Kazakhstan	2.5±0.50*	0.2±0.03*
Average	3.7	0.2
Varieties bred at the Rudnitskii FASC of the North-East		
T-66	0*	0*
C-65	1.7±0.16*	0,1±0,01*
U-80	2.9±0.80*	0,1±0,01*
U-28	4.7±0.95*	0,2±0,03*
C-84	5.1±1.00*	0,2±0,03*
T-123	5.1±1.01*	0,2±0,03*
T-141	5.2±1.00*	0,3±0,05
Bazhenka (standard)	7.4±1.45	0,4±0,15
Average	8.1	0,27
P-57 (reference variety)	21.7±4.40	1,5±0,80

Note. NP — a low-pentose variety.

* Differences from standards (cv. Falenskaya 4 for rye and cv. Bazhenka for wheat) are statistically significant at $P \ge 0.95$.

Table 1 shows cultivars which were the least affected during 3 years of study and are immunologically valuable for rye breeding.

Miedaner et al. [6, 26] found that among four groups of rye plants (population, crossbred, synthetic, and hybrids), population varieties were 2 times more resistant to ergot. Grain contamination with sclerotia was 0.37% for the least affected population variety and 0.89% for a hybrid. There were no significant differences in the composition of EAs [26]. The most common alkaloids were ergosine, ergocristine, and ergotamine. However, their overall content had significant genotypic variability. The phenotypic manifestation of the trait is also associated with the ploidy of the genotype, as evidenced by the studies of rban et al. [3]. According to the authors' report, tetraploid (×4) genotypes which have a longer open flowering period than diploid varieties (×2) are more susceptible to ergot. It can be assumed that diploid rye populations are more promising in breeding for ergot resistance. At present, 109 varieties of winter rye are included in the State Register of Breeding Achievements in the Russian Federation, but there are only 10 hybrids and 9 varieties of the tetraploid type, the rest are diploids. In Germany, on the contrary, hybrids occupies approximately two thirds of all areas for rye cultivation [26].

The ergot resistance of spring wheat, which is mainly due to the short time and closed type of flowering, was significantly higher [25]. Nevertheless, as in rye, significant variability of immunological parameters occurred (see Table 1). In the varieties of the Rudnitskii North-East FASC, the disease prevalence varied from 0 (T-66) to 21.7% (P-57) with 0 (T-66) to 1.5% (P-57) sclerotia in grain batches; for collection accessions, the parameters ranged from 0 (Novosibirskaya 18) to 13.9% (LT-3) and from 0 (Novosibirskaya 18) to 1.3% (LT-3), respectively. The Novosibirskaya 18 variety and the new T-66 line did not develop sclerotia upon artificial inoculation with *C. purpurea* (see Table 1). The absence of sclerotia could be due to genes that control the physiological mechanisms of resistance. Thus, the Novosibirskaya 18 plants rapidly develop in the first half of ontogeny (from germination to flowering, phases 10-69 on the Zadoks scale), and belonged to the early maturing group. The stability of the T-66 line was probably due to its dwarfism and the strength of the straw, which allow the plants to avoid lodging and ensure pollen formation. We classified the varieties Tulaikovskaya nadezhda and Kayir as medium-resistant.

Among the studied winter rye and spring soft wheat gene pools, the collection accessions from Russia and abroad showed the best immunological estimates on resistance to ergot compared to the varieties bred at the North-East FANC. Immune and less affected varieties we selected under a severe infectious load can be the donors of resistance.

The biometric parameters of sclerotia varied significantly in rye and wheat varieties. Thus, the weight of a sclerotium in rye varied from 0.05 (Bereginya NP) to 0.34 g (Grafite) and averaged 0.10 g for collection accessions and 0.20 g for the North-East FANC varieties. In spring wheat, the Chinese variety Long Chun 7 and the line H-154 had the smallest (0.06 g) and large (0.15 g) sclerotia, respectively. Both in rye and in wheat, the largest sclerotia appeared in the varieties bred at the North-East FANC.

The analysis revealed the absence of EA in 9 out of 20 samples of sclerotia collected from winter rye plants and in 4 out of 10 samples from spring wheat (Table 2).

2. Ergot alkaloids in sclerotia of *Claviceps purpurea* (Fr.) Tul. developed on winter rye (S*ecale cereale* L.) and spring soft wheat (*Triticum aestivum* L.) varieties differing in resistance to ergot (*M*±SEM; artificial inoculation, field tests, Kirov, 2017-2019)

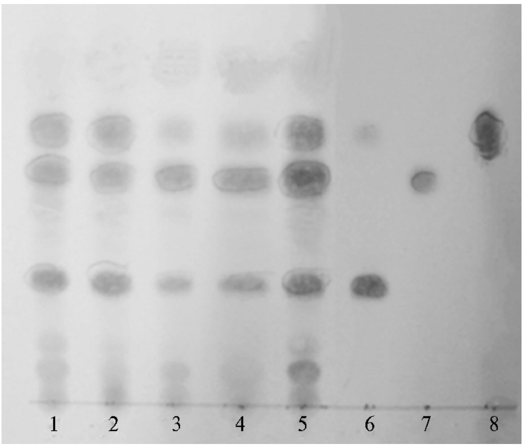
Variety, line	Geographic origin	Ergot alkaloids	
		from sclerotia weight, %	composition
		Winter rye	
Vyatka 2	Kirov Province	0.22±0.009	EA, EM, EC
Falenskaya 4		0.14±0.007	EA, EM, EC
Gratsiya		0.10±0.004	EA, EM, EC
Grafit		0	
Perepel		0	
Rumba		0	
Garmoniya		0	
Simfoniya		0	
Triumph		0.04±0.002	EA, EM, EC
Nioba		0	
Leda		0.17±0.008	EA, EM, EC
Sadko		0	
Rosa		0	
Sara		0	
Amilo 2	Leningrad Province	0.14±0.006	EA, EM, EC
Yantarnaya NP		0.07±0.009	EA, EM, EC
Podarok NP		0.20±0.010	EA, EM, EC
Vavilovskaya NP		0.06±0.002	EA, EM, EC
Rushnik 2 NP		0.36±0.015	EA, EM, EC
Bereginya NP		0.06±0.002	EA, EM, EC
		Spring soft wheat	
N-154	Kirov Province	0	
P-57		0.06±0.002	EA, EM, EC
C-84		0.09±0.004	EA, EM, EC
T-38		0	
T-79		0.24±0.011	EA, EM, EC
Orenburgskaya 23	Orenburg Province	0	
LT-3	Leningrad Province	0.12±0.005	EA, EM, EC
Samgau	Kazakhstan	0.14±0.006	EA
Epos	Germany	0	
Long Chan	China	0.12±0.004	EA, EM, EC

N o t e. EA, EM, and EC — ergotamine, ergotaminine, and ergocristine, respectively.

In other samples, the EA levels varied significantly, e.g., in rye sclerotia from 0.04 (Triumph) to 0.36% (Rushnik 2 NP) of their mass, in wheat sclerotia from 0.60 (line P-57) to 0.24% (line T-79). In our earlier studies [9], the total content of EA in rye sclerotia reached 0.90%, which may be due to excessive moisture during the sclerotia formation. Miedaner et al. [6] also emphasized the close relationship between climatic factors, the EA content and composition. Oeser et al. [18] noted that, with regard to the wide phylogenetic specialization of the biotrophic pathogen *C. purpurea*, it is reasonable to use its various strains isolated from grain taxa.

Correlation analysis revealed a negative relationship ($p = 0.05$) between the total mass of sclerotia and the content of EA (the $r = –0.46$ for rye and $r = –0.32$ for wheat). However, this trend which is important from a practical point of view, requires additional statistical proofing. Considering that small sclerotia cannot be completely separated from grain during mechanical sorting and some of them end up in seed and food grain batches [27-29], the danger of this fraction seems to be the most serious. In turn, the rye grain contamination with sclerotia was significantly (at $p = 0.05$) influenced by the ergot damage to varieties ($r = 0.70$) and the sclerotia weight ($r = 0.69$).

In 17 studied samples of sclerotia, metabolites 1 and 2 were found migrating on TLC with Rf = 0.21 (I) and Rf = 0.49 (II). They fluoresced in UV light ($\lambda = 254$ nm) and developed violet color with Erlich's reagent. The mass spectra of the metabolites were identical and had a negative molecular ion of 580



Thin-layer chromatography of extracts from sclerotia collected on different varieties of winter rye (*Secale cereale* L.) after spraying with Ehrlich's reagent: 1 — Amilo 2, 2 — Yantarnaya NP, 3 — Podarok NP, 4 — Vavilovskaya NP, 5 — Rushnik 2 NP, 6, 7 and 8 — ergotamine, ergocristine, and ergotaminine standards, respectively. Silica gel F_{254} (Merck, Germany), chloroform:methanol:25% NH_4OH (90:10:0,1).

$[M-H]^-$. The chromatographic mobility and MS/MS spectrum of metabolite 1 coincided with the ergotamine standard, and metabolite 2 with the ergotaminine standard. Therefore, the metabolites 1 and 2 were identified as the peptide ergoalkaloids ergotamine and its stereoisomer ergotaminine. In the same samples of sclerotia, metabolite 3 with Rf = 0.41 (I) was found which also fluoresced and produced violet color with Ehrlich's reagent. MS/MS of the metabolite 3 had a negative molecular ion 608 $[M-H]^-$. The chromatographic mobility and MS/MS spectrum of metabolite 3 matched the ergocristine standard. Therefore, we identified the metabolite 3 as ergocristine (Fig.).

In rye, the correlation between the ergot damage to plants and the total EA content was $r = 0.22$ ($p = 0.05$), which indirectly indicates the absence of a significant relationship between the toxicity and pathogenicity of the fungus *C. purpurea*. Nevertheless, data on the content of ergoalkaloids is important for the search for genotypes of grain crops with the lowest accumulation of EA that makes sclerotia a less dangerous mechanical impurity in the grain mass. The varieties that combine resistance to ergot damage and lower EA accumulation are of the greatest breeding and immunological value. Among them are the German variety of spring wheat Epos and new populations of winter rye Symfonia, Garmonia and Rumba. The high-yielding ergot-resistant cv. Rumba is ready for the state testing. The Garmonia population was obtained using donors of resistance to ergot and fusariosis of the ear [1].

Thus, qualitative and quantitative analysis of ergoalkaloids (EAs) upon artificial inoculated with *Claviceps purpurea* showed that in sclerotia of five wheat varieties and eleven rye varieties the EAs were ergocristine, ergotamine and its stereoisomer ergotaminine. In the Kazakh wheat variety Samgau, we identified only ergotamine. The content of EAs in sclerotia differed significantly. Varieties of winter rye and spring wheat that do not accumulate EAs in sclerotia are of practical importance, since the contamination of food and feed grains with such sclerotia does not pose a biological hazard. Genotypes that combine resistance to ergot damage with the absence of EA accumulation in sclerotia (e.g., the winter rye Rumba, Sympfoniya, Garmoniya and spring wheat Epos) are of particular breeding value as donors of these traits.

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