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THE POTENTIAL FOR EVOLUTION OF HEAVY METAL TOLERANCE IN PLANTS

III. THE RAPID EVOLUTION OF COPPER TOLERANCE IN *AGROSTIS STOLONIFERA*

LIN WU*, A. D. BRADSHAW and D. A. THURMAN
Department of Botany, University of Liverpool

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SUMMARY

A series of grasslands of different ages which have suffered copper pollution for different lengths of time have been used to follow the evolution of metal tolerance.

Species diversity in the grasslands decreases with increasing pollution, so that in the most polluted sites only *Agrostis stolonifera*, sometimes with *Agrostis tenuis*, is to be found. In the polluted sites there is a great deal of bare ground in the youngest (5 years old) grasslands but complete cover in the oldest grasslands (70 years old).

The *A. stolonifera* populations in unpolluted sites are not copper tolerant. But in the polluted sites there is an increase in the tolerance of individuals, and an increase in the frequency of tolerant individuals, as the age of the population increases: even the youngest population shows considerable tolerance.

An analysis, using morphological and esterase isoenzyme variation as criteria, shows that the tolerant populations consist of a large number of different individual clones or genotypes. The populations must have evolved by the rapid selection of a large number of the tolerant or partly tolerant individuals which can be shown to exist in unpolluted populations at very low frequency.

The selection can be shown to occur at both seedling and adult stages, and to cause the evolution of tolerance effectively in a single generation.

1. INTRODUCTION

THE geographical spread of aerially borne heavy metals has been extensively studied by analysis of their accumulation in plants and soils (Goodman and Roberts, 1971, and others). Because these metals can be extremely toxic to plants in low concentration, plants are often the initial indicators of a pollution problem, such as exists in areas surrounding heavy industries (Heck, 1966).

Such aerial pollution can be severe. As a consequence, although it is often of recent origin, there is no reason why it should not cause significant changes in the genetic structure of the plant populations exposed to it, in the same way that terrestrial pollution from mining for metals has caused genetic changes (Antonovics, Bradshaw and Turner, 1971) which can occur rapidly (Walley, Khan and Bradshaw, 1974).

A metal refining industry was established about 1900 at Prescot in S.W.

* Present address: Department of Botany, Duke University, North Carolina.

Lancashire: it now continues as part of British Insulated Callenders Cables Ltd. The refineries have caused considerable aerial pollution on the surrounding vegetation. The major constituent in the dust particles emitted by the chimneys is copper, but there is also some lead and zinc. Grasslands near the refineries have now a total soil copper content of up to 4000 p.p.m., and in some places the vegetation has been totally destroyed. The maintenance of lawns in the immediate vicinity of the refineries has usually depended on the surface soil being changed at regular intervals. Where there is established grassland it is dominated by *Agrostis stolonifera* or *Agrostis tenuis* and few, if any, other species.

Within the grassland areas at Prescot the populations of *A. stolonifera* have different structures which seem to be related to their age. In populations less than 10 years old there are large amounts of bare ground and the plants occur as individual patches. But in older populations there is a continuous plant cover. In all these areas the metal levels are so high that it is unlikely that normal non-tolerant plants could grow. It therefore seemed possible that these lawns represent an evolutionary series in which tolerant plants are being selected and are spreading both by seed and vegetatively to form new tolerant populations. If this was so, it would provide a remarkable opportunity to study evolution in action.

2. Surveys of soil and plants

Nine sites of different ages and with different population structures in contaminated areas near the copper refinery, and two sites in uncontaminated environments, were chosen for detailed study.

(i) *Materials and methods*

Soil and plant analyses were carried out to obtain a direct measure of pollution. Three replicates of soil samples and plant materials were collected at random from each site. Plants were collected with their roots and associated soil in a tuft 8 cm × 8 cm × 3 cm deep. The plants were washed by three changes of 0·2 per cent Teepol solution and then washed three times in deionised water. The soil and plant materials were dried at 60° for two weeks. The total heavy metals were extracted by boiling 1 g of dried soil sample with 25 cm^3 concentrated nitric acid for one hour. The sample was then filtered and washed with deionised water until the filtrate became colourless. The final volume of the filtrate was made up to 100 cm^3.

The water-soluble heavy metals were extracted by shaking a 2·5/1 water/soil mixture for 6 hours at room temperature. The suspension was then filtered and centrifuged and the clear extracts used for the determination of heavy metals.

The dried plant materials were ground finely. 1 g of the material was extracted with 25 cm^3 of 6N hydrochloric acid boiled gently for 15 minutes: after filtering this was made up to 100 cm^3. The metals were determined by using SP 90 atomic absorption spectrophotometer.

The age of the plant population of each site was determined from historical records. It was defined as the time since the lawn or grassland was established in polluted conditions. The structures of the different populations were determined by visual inspection.

(ii) *Results*

The nine communities in the copper refinery area are very different from each other in their structure (table 1). At one extreme they are com-

TABLE 1

The structure and age of the plant communities of the nine sites in the vicinity of copper refineries and two sites in uncontaminated environments

Site	Age	Plant cover	*A. stolonifera*	Other species
Flowerbed new lawn*	8	Isolated patches	Abundant	None
Flowerbed old lawn*	70	Continuons	Abundant	*A tenuis* only
Old lawn*	70	Continuous	Abundant	*A. tenuis* only
New lawn adjoining the new refinery	4	Almost destroyed	Rare	Several
New lawn near the new refinery	4	Continuous	Frequent	Several
Boundary grassland*	4	Fairly continuous	Frequent	Several
Canteen lawn*	14	Coalesced patches	Abundant	*A. tenuis* only
Computer building new lawn	3	Continuous	Frequent	Several
Recreation ground grassland	20	Isolated or coalesced patches	Frequent	*A. tenuis* only
Sefton Park grassland*	—	Continuous	Occasional	Many
Freshfield sand-dune grassland*	—	Continuous	Frequent	Many

* Populations used for further studies.

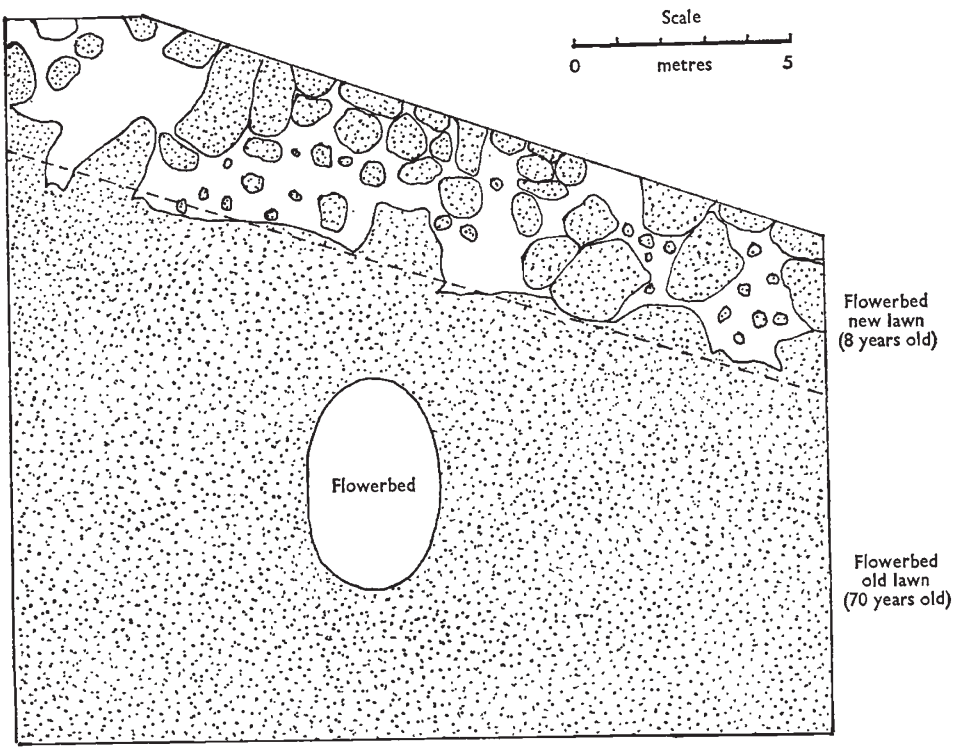

FIG. 1.—Map of the structure of the Flowerbed new lawn and the adjacent Flowerbed old lawn.

posed of isolated patches, at the other of a completely continuous vegetative cover; these can occur side by side as in the Flowerbed lawns (fig. 1). The species diversity, although not recorded in detail, also varies from one site to another. The New lawn near the new refinery, and the Boundary grassland, have a wide diversity of species, whereas the Old lawn and the

TABLE 2

Distribution of copper, zinc and lead in the soil from the different sites (p.p.m.)

		Metal content of soil						
		Available			Total			
Site	*p*H	Cu	Zn	Pb	Cu	Zn	Pb	Copper content of plant materials
Flowerbed	4·8	24	26	—	5300	740	600	800
new lawn	5·2	20	18·5	—	4900	740	740	360
	5·4	34	20	—	4300	500	500	1000
Flowerbed	5·2	24	20	—	4400	700	600	1100
old lawn	5·3	30	25	—	4000	600	400	400
	5·2	40	20·5	—	4200	750	600	600
Old lawn	6·8	18	12	—	3800	280	700	400
	6·5	20	9·5	—	3600	320	600	640
	6·5	22	8	—	5400	320	600	640
New lawn	5·2	30	8	—	9500	300	140	1100
adjoining the	5·6	25	8	—	10500	400	140	820
new refinery	5·2	35	3	—	10800	150	130	600
New lawn	6·7	4	8	—	720	120	140	300
near the new	6·5	6	6	—	1800	600	150	610
refinery	6·5	4	6	—	1080	600	140	100
Canteen lawn	5·5	18	14·5	—	2000	700	130	400
	5·1	20	18	—	2400	500	130	350
	5·5	19	16	—	3500	300	130	600
Computer	6·0	6	8	—	300	200	140	600
building	6·5	4	6	—	300	300	150	420
new lawn	6·0	4	10	—	350	350	130	310
Boundary	4·6	12	4	—	2230	150	100	—
grassland	5·0	10	3	—	2060	150	100	—
	5·1	14	4	—	1500	200	200	—
Recreation	4·0	12	8	—	1800	100	200	420
ground	5·5	20	8	—	3100	250	130	750
grassland	4·5	19	12	—	2210	80	250	520
Sefton Park	6·6	0·6	0·3	—	70	70	70	20
grassland	6·6	0·8	1·4	—	90	80	40	18
	5·2	0·9	2·5	—	40	70	30	10
Freshfield	7·6	0·8	1·4	—	20	60	30	19
sand dune-	7·3	0·7	1·0	—	40	60	30	21
grassland	7·4	0·8	0·8	—	40	60	20	10

Flowerbed new lawn are dominated by two or one species of *Agrostis* without any other species being present. Both uncontaminated sites had continuous cover and a wide variety of species.

Copper is the major heavy metal contaminant in the soil (table 2). The total copper content is about 2000 p.p.m. in the low-contaminated sites and about 4000 p.p.m. in the higher contaminated sites, but only about 50 p.p.m. in the uncontaminated sites. In some places in the immediate

vicinity of the copper refinery the total copper content in the surface soil is up to 10,000 p.p.m. The water-soluble copper content in the soil correlates with the total copper content.

The total zinc and lead contents in the soil of the nine sites of the copper refinery area are also higher than in the soil of the two uncontaminated sites. The occurrence of zinc can be explained by the existence of a brass foundry on the site. The lead is probably due to the now obsolete practice of covering the electric cables manufactured on the site with a lead sheathing.

The copper content in the plant materials which were collected from the sites of the copper refinery area are from 100 p.p.m. to 1000 p.p.m.; the copper content in the plant materials from Freshfield and Sefton Park are about 20 p.p.m.

(iii) *Discussion*

By any standard the amount of copper in the soils of the refinery sites is very high. In comparison the soils of Parys Mountain, Anglesey, and Drws-y-Coed, Caernarvonshire, copper mines contain about 1800 to 2600 p.p.m. copper. These are known to cause powerful selection on plants (McNeilly and Bradshaw, 1968). Thus it is reasonable to believe that the copper is causing powerful selection on plant populations at Prescot. In some sites the total zinc content is up to 700 p.p.m., and the water-soluble zinc content is up to 26 p.p.m. But this level of zinc in the soil is much lower than that of copper and it is known that the zinc is less toxic to plants than copper. Thus it is likely that copper is playing the most significant selective role. Whether the zinc is having any effects was not investigated. The lead levels are below those that would cause toxicity.

The structure of the different grasslands in the vicinity of the copper refineries are related to the metal contamination. The reduction in the number of plant species with contamination is very similar to the changes in plant communities on copper-mine workings (McNeilly and Bradshaw, 1968).

The structure of the populations is also related to their age. This is well shown by the Flowerbed new lawn, Canteen lawn, Flowerbed old lawn and Old lawn, which are of different ages but are all on heavily contaminated soils. Vegetative cover in these populations is related to their age. This could be due to selection for metal-tolerant genotypes. In the youngest population, the Flowerbed new lawn (8 years), the patchiness suggests that the process is only partly complete. In the Canteen lawn, which is slightly older (15 years), individual patches had been observed to coalesce only a few years previously: perhaps here the evolution of tolerance is just complete.

3. Copper tolerance

These observations suggest that copper tolerance must be evolving at Prescot and that the structure of the various populations arises by the interaction of the copper contamination and the evolution it has caused. It was therefore necessary to examine in detail the tolerance of all these populations to see whether this deduction was correct. The most appropriate test for this is the now well-established rooting test.

The soil in the vicinity of copper refinery must have become contaminated by copper only after the first copper refinery was established. We do not know the origin of the populations of *A. stolonifera* in the copper refinery except that they were all either sown with commercial seed or established from normal uncontaminated turf: in which case none of the populations would have been tolerant before the establishment of the refinery. However, the copper tolerance must have come from these non-tolerant populations.

A sensitive method to reveal the existence of genes for metal tolerance in non-tolerant populations is by sowing the seeds of non-tolerant populations on metal-contaminated soil (Walley, Khan and Bradshaw, 1974). Thus a seed test of this sort was made to investigate the potential for the evolution of copper tolerance from non-tolerant populations.

In the one very young site, the Flowerbed new lawn, the individual patches, which appeared to be different clones, varied in size (fig. 1). It seemed possible that this could be due to differences in tolerance (although it could equally be due to differences in age). This population was therefore subject to a separate investigation.

(i) *Materials and methods*

(a) *Sampling*

From five of the populations of *A. stolonifera* in the copper refinery area and the two populations in uncontaminated environments 30 tillers were collected at intervals of 20 cm along a transect or two parallel transects across each site. These were then propagated in normal soil in a glasshouse for at least 8 weeks prior to testing for copper tolerance.

To examine the tolerance of individual clones of different size on the Flowerbed new lawn, three tillers were taken from each of 15 apparently individual plants. These were propagated as before in normal garden soil prior to testing.

To examine the possible processes of selection for copper tolerance in seed populations, seed was collected from six sites.

(b) *Copper tolerance test*

Two methods were used for measuring tolerance both involving rooting tests:

(*a*) The parallel method described by Jowett (1964) and modified by McNeilly and Bradshaw (1968). This method was used for measuring copper tolerance of all seven populations. For each plant 10 tillers of comparable size and age were suspended in a solution containing calcium nitrate at 0·5 g/litre, and a further ten in a solution containing calcium nitrate and Cu^{++} as copper sulphate at 0·126 p.p.m., at 25° with continuous illumination. The individual plants were fully randomised. The solutions were changed every 2 days to provide aeration and to maintain the copper concentration. After 10 days the longest root of each tiller was measured and the index of tolerance calculated:

$$\text{Index} = \frac{\text{Mean length of longest root in solution with copper}}{\text{Mean length of longest root in solution without copper}}$$

An analysis of variance and Duncan's new multiple range test were used for the statistical test of the population means, in which the 30 individual mean indices of copper tolerance of the 30 individual plants of each population were treated as replicates.

Prior to this experiment the behaviour of three plants was examined at five different copper levels using the same method.

(*b*) The series method devised by Wilkins (1957) was used in a second experiment involving the Freshfield sand-dune grassland, the Boundary grassland population and the Old lawn population. To look at these in more detail there were three replicates, with four tillers of each of the 30 plants of each population in each replicate.

Tillers were suspended as before in calcium nitrate solution which was changed every second day. After 6 days the growth of the longest root of each tiller was measured over a 2-day period. The tillers were then transferred into a solution containing calcium nitrate (0·5 g/l) and copper (0·063 p.p.m.) as copper sulphate; because the root growth of the non-tolerant plants was too slow to be measured in 2 days, the growth of the longest root of each tiller was measured after a 4-day period. The index of copper tolerance was calculated as before allowing for the longer period in copper solution.

The statistical analysis was made on the three populations together, using Duncan's multiple range test and a separate analysis on each population to determine differences between plants within populations.

In the preliminary test it was found that 0·126 p.p.m. copper was the optimum concentration in the parallel method to distinguish the different degrees of copper tolerance. But in the series method the copper concentration had to be reduced to 0·063 p.p.m. Despite this adjustment it must not be expected that the different methods give identical absolute results but the two tests should give similar relative results.

(c) *Seed experiment*

To test the occurrence of tolerant individuals at very low frequency, seed samples were sown on contaminated soil. Two thousand seeds of each population were sown in a seed tray on copper-contaminated soil from the refinery containing 10,260 p.p.m. Cu. The seed trays were kept in a glasshouse and watered every second day with tapwater. After 9 months the number of surviving plants growing on the contaminated soil which had more than three tillers were counted. The copper tolerance of these plants was tested, using the parallel method.

(ii) *Results*

Marked copper tolerance, of different degrees in different plants, was found in the populations. The relationship between the root growth and the copper concentration of three selected plants with different degrees of copper tolerance in the preliminary experiment are plotted in fig. 2.

The distributions of copper tolerance in the seven populations in the first test are given in fig. 3. The copper tolerance of individuals of the Freshfield population and the Sefton Park population are uniformly low. In the population on the boundary of the refinery area there is a considerable increase in the occurrence of tolerance, the tolerance being more or less

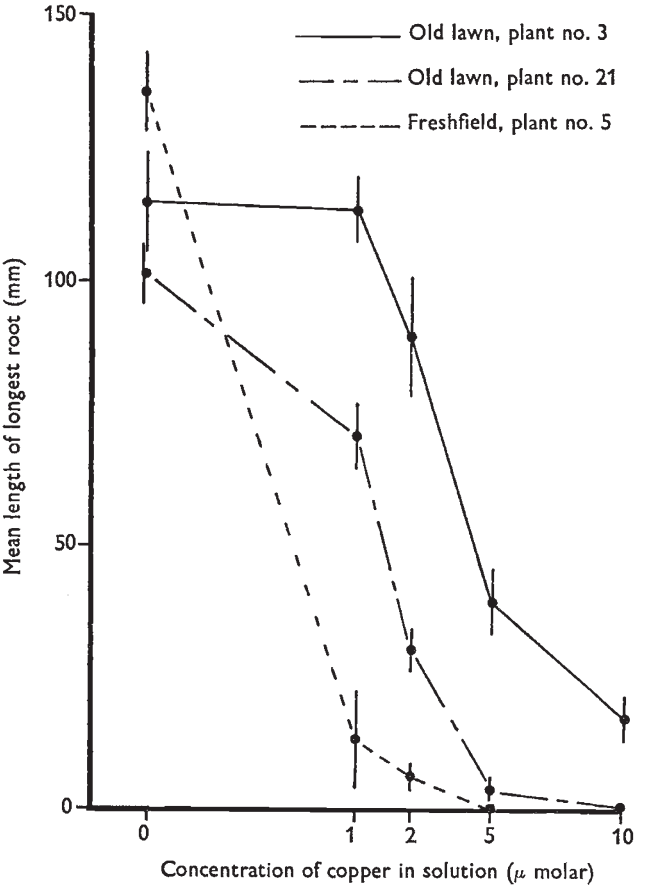

FIG. 2—The effect of copper concentration on the rooting of three different plants of *Agrostis stolonifera*

continuously distributed. In the Flowerbed new lawn, Canteen lawn, Flowerbed old lawn and Old lawn populations there is a further progressive increase in mean tolerance (table 3).

The distribution of copper tolerance in the three populations in the

TABLE 3

Differences in population means of copper tolerance of seven populations of A. stolonifera (*parallel method*)

Population	Freshfield sand-dune grassland	Sefton Park grassland	Boundary grassland	Flowerbed new lawn	Canteen lawn	Flowerbed old lawn	Old lawn
Mean index of copper tolerance	0·07	0·06	0·21	0·32	0·42	0·46	0·53
Duncan's new multiple range test (5% prob.)	———				———	———	

second test using the series method (fig. 4) gives similar results. The analyses of variance for the three populations (table 4) shows that the mean squares between the populations are greater than the mean squares between the individual plants within the populations. There is no significant difference

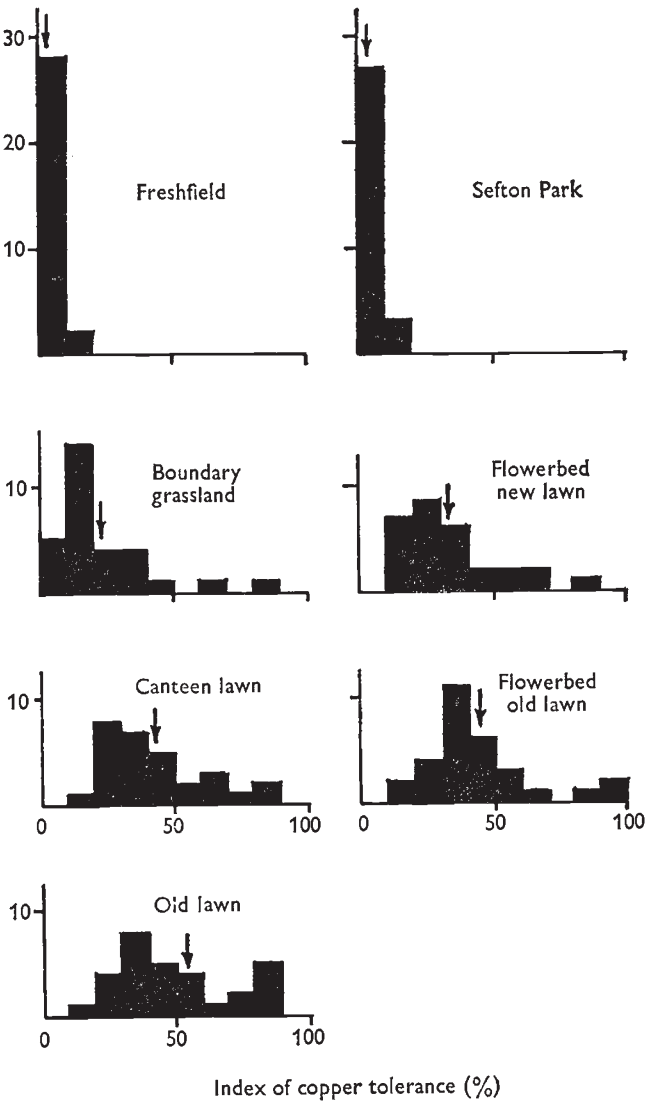

FIG. 3.—Distribution of copper tolerance in the samples of seven populations of *Agrostis stolonifera* (parallel method) (means denoted by arrows).

between individual plants within the Freshfield sand dune population, but considerable difference between individuals within the Boundary grassland and the Old lawn populations.

A correlation analysis of the two sets of copper-tolerance values of the

three populations which were measured by both methods showed that the two sets of indices of tolerance for the Boundary grassland population and the Old lawn population are highly significantly correlated: the correlation coefficients are 0·67 and 0·75 respectively. The two sets of values for the Freshfield sand dune population do not show correlation, almost certainly because they are uniformly low and do not spread.

The relationship between the copper tolerance of the plants collected from the Flowerbed new lawn and their size is shown in fig. 5. The correla-

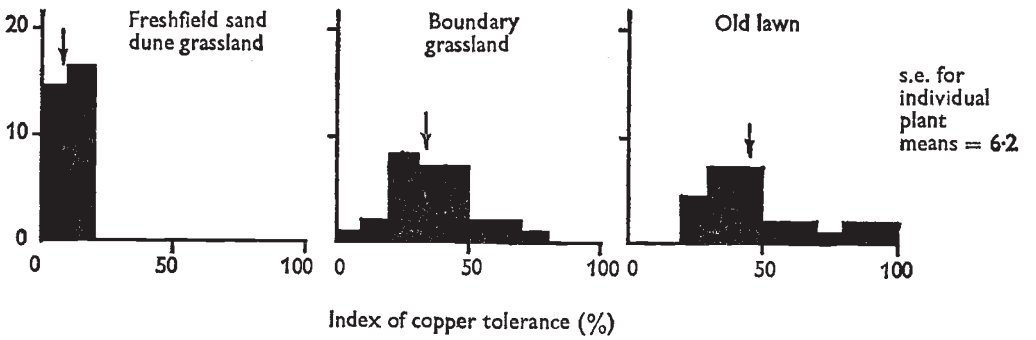

FIG. 4.—Distribution of copper tolerance in the samples of three populations of *Agrostis stolonifera* (series method) (means denoted by arrows).

TABLE 4

Analysis of variance of copper tolerance in three populations (series method)

Source		MS	DF	F	Significance level
Between populations		3·62321	2	309·2	***
Between plants within populations	Freshfield dune grassland	0·00097	29	0·83	n.s.
	Boundary grassland	0·06491	29	5·5	***
	Old lawn	0·18303	29	15·6	***
Between blocks		0·00283	2	0·24	n.s.
Error		0·01172	178	—	—
Total		0·06155	269	—	—

tion coefficient between plant size and index of copper tolerance is significant at 5 per cent level.

In the seed-screening test almost all the seedlings in non-copper-contaminated seed samples growing on the copper-contaminated soil were dead after 9 months. However, a very few had survived and produced three or more tillers. There were two plants in the Freshfield seed population, one plant in the Trelogan zinc and lead mine population and two plants in the Halkyn mountain lead mine population. Unfortunately, the plant surviving in the Trelogan mine seed sample was lost. The remaining four plants were tested for their copper tolerance. The results show that they were all copper tolerant but at an intermediate level (table 5).

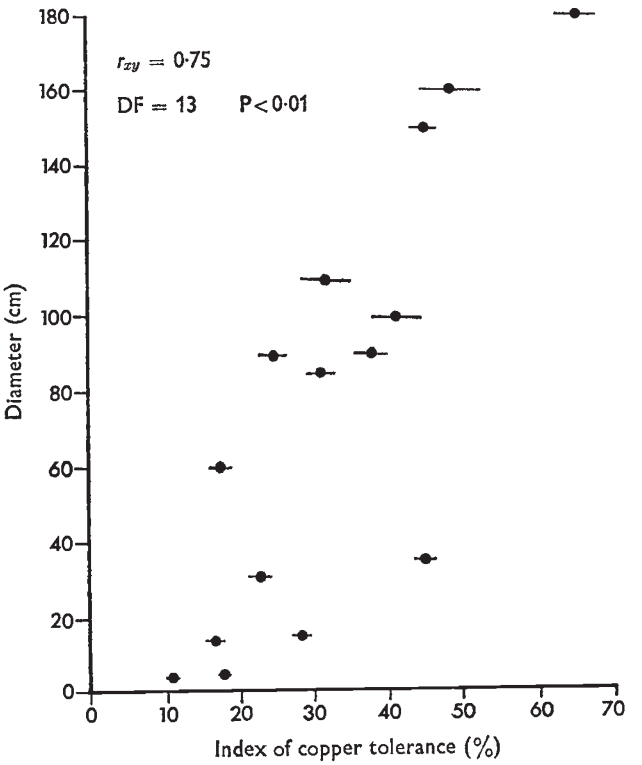

FIG. 5.—The relation between size and copper tolerance of 15 plants collected from the Flowerbed new lawn.

TABLE 5

The results of selecting for copper tolerance in seed populations

Seed source	Number of seedlings grown to tillering stage in refinery soil (out of 2000)	Index of copper tolerance
Freshfield sand-dune grassland	2	0·18
Trelogan zinc and lead mine	1	*
Trelogan pasture	0	—
Halkyn Mountain lead mine	2	0·22
		0·26
Grassland near canteen lawn (refinery)	82	*
Boundary grassland (refinery)	94	*

* Seedlings not tested.

(iii) *Discussion*

It is clear that copper tolerance has evolved in the *A. stolonifera* populations at Prescot, and that the genetic structure for copper tolerance of the

populations in the refinery area is related to the intensity of the contamination and the length of time during which the vegetation has been contaminated. In the Boundary population, which is the most recently contaminated, there is a considerable increase over the control populations in the occurrence of copper-tolerant individuals, tolerance being more or less continuously distributed. This suggests that many different genotypes have been selected for copper tolerance from an original non-tolerant population and that this selection is occurring now.

The further progressive increase in the mean copper tolerance and the disappearance of non-tolerant and slightly tolerant individuals in the Flowerbed new lawn, Flowerbed old lawn, Canteen lawn and Old lawn, represents a historic series of the evolution of copper tolerance.

At the outset all individual genotypes except those which had some degree of tolerance must have been eliminated, and then the tolerant genotypes must have spread to close the sward. Since there is an increase in mean tolerance there must also have been progressive selection for increasingly tolerant genotypes perhaps produced by recombination from interbreeding among the original individuals.

The Flowerbed new lawn and the Flowerbed old lawn lie side by side. It is known that the Flowerbed new lawn has been formed by the repeated sowing of a standard seed mixture of unknown composition but certainly not metal tolerant. The differences in the distribution of copper tolerance between the Flowerbed new lawn and the Flowerbed old lawn populations suggest that the original copper-tolerant plants of the Flowerbed new lawn must have been independently selected and have not arisen by simple " infection " by copper-tolerant individuals from the Flowerbed old lawn. The great difference in vegetative structure of the two lawns is then a sign of the different degree of development of a copper-tolerant plant population.

The second set of tolerance tests on the three selected populations show good agreement with the first. The variation between plants in the two contaminated populations is highly significant. They are certainly not uniform.

The screening experiment shows that the potential to evolve metal tolerance is present in the populations of *A. stolonifera* from Freshfield and other areas not contaminated with copper. Although most seedlings died, a very few survived well, and were found to be tolerant. From other work (Gartside and McNeilly, 1974) it seems certain that they would give rise to tolerant offspring and eventually to a fully tolerant population. Such individuals did not show up in the previous tiller tests, almost certainly because of the restricted sample size. Selection can therefore be very effective in causing evolutionary change by acting on seedling stages of *A. stolonifera*, and this must have occurred at Prescot.

The copper tolerances of the 15 clones in the Flowerbed new lawn were significantly correlated with the size of the individual clones. The clones which were vegetatively the most successful were the more tolerant. This implies that the selection at Prescot is not a single-stage process but a two-stage one acting on adults as well as seedlings. Selection operates ultimately through the capacity to leave descendants. This depends firstly on survival but also secondly on growth and reproduction subsequently. Very little evidence of selection at the second stage exists for plants, not even in detailed work such as that of Charles (1961).

4. Genotypic structure of the populations

A. stolonifera is a vigorous perennial capable of spreading vegetatively at least 1 metre per year by means of stolons. All the sites examined were either grazed or mown. Under these conditions the populations could have developed by the spread of a very few initial colonists and therefore may now be composed of only a very few clones and therefore individual genotypes. This possibility has been clearly demonstrated by the extensive work of Harberd (1961, 1967).

The youngest population, the Flowerbed new lawn, is apparently composed of many different individual plants. Whether they are all members of a single clone which has been spread vegetatively is not clear. The older populations could certainly be derived from the growth of a very few tolerant clones. Even if a number of clones were established in the first instance, the competition in the closed sward, coupled with the severity of the environment, could well have resulted in the ultimate dominance of a few clones in the sward and the elimination of the rest.

It was therefore necessary to examine the clonal structure of the swards, to see whether the populations were composed of only one or a few genotypes. Two techniques for identification of clones were used, one based on morphological differences, the other based on isoenzyme differences.

Morphological methods of recognising clones, and therefore individual genotypes, in natural populations of grasses was initiated by Harberd (1958), and applied to hybrid populations of *Agrostis* by Bradshaw (1958). There are many morphological attributes which can be used which allow a fairly precise identification of different clones. Although the differences are not always easy to record quantitatively they are usually easy to determine by eye.

The recognition of clones by a determination of their isoenzyme complements has not previously been attempted. However, the existence of isoenzyme variation and its use in evolutionary studies is well established (review by Gottlieb, 1971). Most of the work has been carried out on animals, but there is now good evidence for similar variation in plants (*e.g.* Marshall and Allard, 1970).

The particular advantages of using isoenzyme variation for the recognition of clones is (*a*) its simplicity and (*b*) its precision. There is no need to carry out a genetic analysis of the variation: it is sufficient to show that it has definite identity. Its main disadvantage is the limited amount of variation available which may not provide sufficient distinguishable genotypes to allow the recognition of all the different clones that actually exist in a population. However, the choice of proteins showing considerable variation should enable at least the same fineness of separation as can be got by the use of morphological differences. For this reason the esterase enzyme was chosen for this study: it shows considerable variability in *A. stolonifera*. Certainly the method provides the same sort of precision as the use of incompatibility characters which was very effective in *Festuca rubra* (Harberd, 1961), but with less labour.

(i) *Materials and methods*

(a) *Populations*

The five populations were studied, the Flowerbed new lawn, the Canteen lawn, the Old lawn, the Freshfield dune grassland and the Sefton Park

grassland. A sixth population, from the face of a sea cliff at Abraham's Bosom in Anglesey, was also included for isoenzyme studies. It had been collected previously from a small area of exposed seacliff: 30 plants were picked from the collection at random. It was included to provide another control, uncontaminated, population.

An additional sampling was made in the Canteen lawn. This lawn had become completely covered by grass only recently and the boundaries of different clones could be seen. Thus it provided good opportunity to look at the process of population development and the spatial patterns produced by vegetative reproduction. Thirty tillers were taken in order at 20 cm intervals in a transect across the lawn. The apparent extent of separate clones were carefully noted on collection. This material was not only examined for isoenzymes but also for metal tolerance.

For morphological examination the five original population samples were planted out together in a randomised block arrangement in the University Botanic Gardens in September 1971, all the material being represented in each of three replicates: the plants were spaced 1 metre apart. For isoenzyme analysis a representative of each plant of the five populations and of the two further collections were grown in a warm greenhouse for 3 months prior to analysis.

(b) *Morphological technique*

The morphological characteristics were studied in July 1972, when the plants were in full flower. The individual plants in each population were grouped by examining the differences in the appearance of the plants. Each individual plant was compared with all the other plants of the same population. From this the plants were placed into groups of identical individuals A, B, C and so on. The three replicates of each population were observed in the same way. If there was any disagreement between replicates, then checks were carried out before the final groupings were made. The matching is not very difficult (Harberd and Owen, 1969). No comparisons were made between populations because it was difficult to compare the 150 individual plants of the five populations in the field by eye, and it was the number of distinct individuals within each population rather than between populations which seemed the most important character.

(c) *Isoenzyme technique*

Crude enzyme extracts were made by homogenising 100 mg of fresh material, of the three uppermost leaves without their ligules, at 4° in a glass mortar with 0·5 ml of 0·01M Tris-HCl buffer (*p*H 6·5), containing 5 per cent (w/v) sucrose. The homogenates were centrifuged at 20,000 g for 30 min, and the clear supernatants used for enzyme assay immediately or stored in a deep freeze overnight.

The polyacrylamide disc-electrophoresis was carried out essentially as described by Davis (1964).

Esterase activity was detected by preincubating gels at 5° in 0·5M boric acid for 2 hours to lower the *p*H to approximately 6·5 and then incubating for 2 hours at 25° in 10 cm^3 tubes with 6 cm^3 of 0·1M phosphate buffer *p*H 6·5 containing naphthylacetate FR (2 mg/100 cm^3) Fast Red TR (50 mg/100 cm^3).

(ii) *Results of morphological analysis*

Distinct clones could be recognised in nearly all populations. The individuals from the refinery populations tended to be remarkably different from each other, particularly with respect to the size and shape of their panicles. They were often also different in the arrangement and general

TABLE 6

Clones in populations of Agrostis stolonifera *recognised by morphological technique*

Population	Clones distinguished	Number of plants
Freshfield sand-dune grassland	fA	28
	fB	2
Sefton Park grassland	sA	25
	sB	5
Flowerbed new lawn	nA	5
	nB	2
	nC	3
	nD	4
	nE	5
	nF	6
	nG	1
	nH	1
	nI	3
Canteen lawn	cA	7
	cB	4
	cC	2
	cD	7
	cE	5
	cF	2
	cG	1
	cH	1
	cI	1
Old lawn	oA	3
	oB	5
	oC	2
	oD	5
	oE	7
	oF	2
	oG	2
	oH	1
	oI	2
	oJ	1

characters of their stolons: in particular, some plants had erect shoots while others had prostrate ones. The uncontaminated populations looked much more uniform.

In the final analysis (table 6) there appeared to be only two groups, and therefore clones, in both the Freshfield and the Sefton Park populations: the only difference observed between these clones was in the thickness of their stolons. By contrast, in the refinery populations, nine groups were

identified in the Flowerbed new lawn and Canteen lawn and 10 groups in the Old lawn populations.

This suggests that the genotypic structures of these populations are very different. There appears to be very little variation within the Sefton Park and Freshfield populations: these two populations appear to be made of only one or two individual genotypes. By contrast, the highly selected refinery populations seem to consist of a large number of different genotypes. This was sufficiently interesting to make it essential that the structure of these populations was looked at by an independent method.

(iii) *The results of isoenzyme analysis*

The distinction of different clones was based on the recognition of different esterase zymograms due to the presence or absence of different esterase isoenzymes. It can be assumed that the different esterase bands represent products of different genes, although without breeding experiments, which were not carried out, the exact genetic control of each band cannot be determined.

TABLE 7

Frequency of different esterase zymogram types in the Abraham's Bosom population of Agrostis stolonifera

Arbitary symbol for esterase zymograms	A	B	C	D	E	F	G	H
Number of plants	10	4	1	1	2	3	4	3

There were a few vague bands whose indeterminate nature made recognition difficult: these were omitted. Analysis of different parts of a single plant and different plants from the same clone showed that there were significant and consistent differences between different plant parts, but that isoenzyme patterns were precisely repeatable for any particular plant part, even in material grown in different conditions. There is obviously developmental control of esterase isoenzymes, but even so genetic variation between plants exists and is not masked (Wu and Thurman, 1975).

A summary of the frequency of occurrence of different esterase zymograms of the 5 populations is given in fig. 6. In the Freshfield sand-dune population only one type of esterase zymogram was found. In the Sefton Park population there were two. In the three refinery populations there were nine types of esterase zymogram in the Flowerbed new lawn, 10 in the Canteen lawn and 15 in the Old lawn population. Thirty-four different esterase zymograms were found within the five populations taken as a whole. Only one type of esterase zymogram (zymogram 2) was found in more than one population: it occurred in the Sefton Park population and the three refinery populations. There was not, apparently, any single dominant esterase zymogram type in the refinery populations.

The esterase isoenzymes of the Abraham's Bosom cliff population were analysed later on 28 plants (table 7). Eight different esterase zymogram types were found. Type A was commonest, occurring in over 30 per cent in the plants sampled. The rest of the seven esterase types were much less common. This population is therefore not at all like the other uncontaminated populations.

In the detailed transect of the Canteen lawn, 13 different types of esterase zymograms were found among the 11 clonal patches which could be identified visually. Thus eight of the original clones determined by eye were confirmed by the esterase isoenzyme analysis. However, three of the original clones, A, G and K, are apparently mixtures of different clones (plate I).

The copper tolerance of the 30 plants from the Canteen lawn were measured by the parallel method. Only five tillers were put into copper

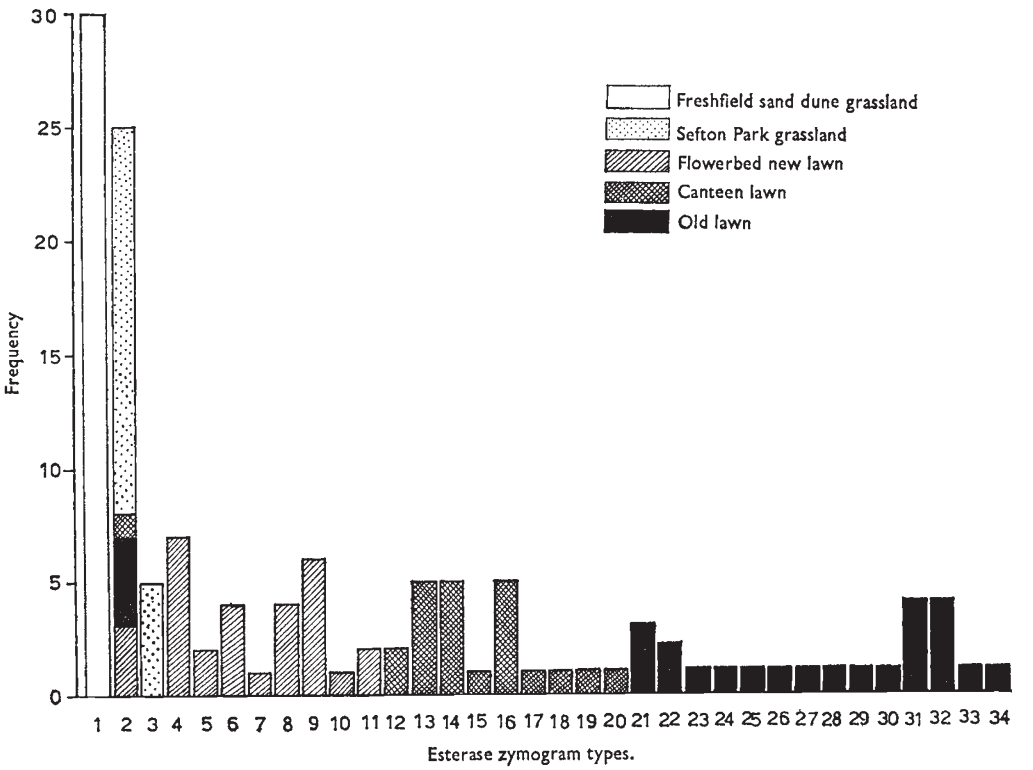

FIG. 6.—Frequency of occurrence of different esterase zymogram types in the samples of five populations of *Agrostis stolonifera* (values for type 2 overlapped).

solution and five into calcium nitrate because of limitations in the number of tillers which had been produced. But the results show that there is similarity in the values for tolerance of different plants which are members of the same clone. It is clear that the Canteen lawn is made up of a number of distinct clones most of which are limited in their spread.

(iv) *Discussion*

The results indicate that there is a considerable variation in esterase zymograms between individual plants taken from the refinery populations and little variation in the two original control populations, and that this complements the findings based on morphology. There are some dis-

crepancies between the two techniques (fig. 7) in that groupings based on morphological similarity may be subdivided on isoenzyme characters, or vice versa; but the general picture is clear.

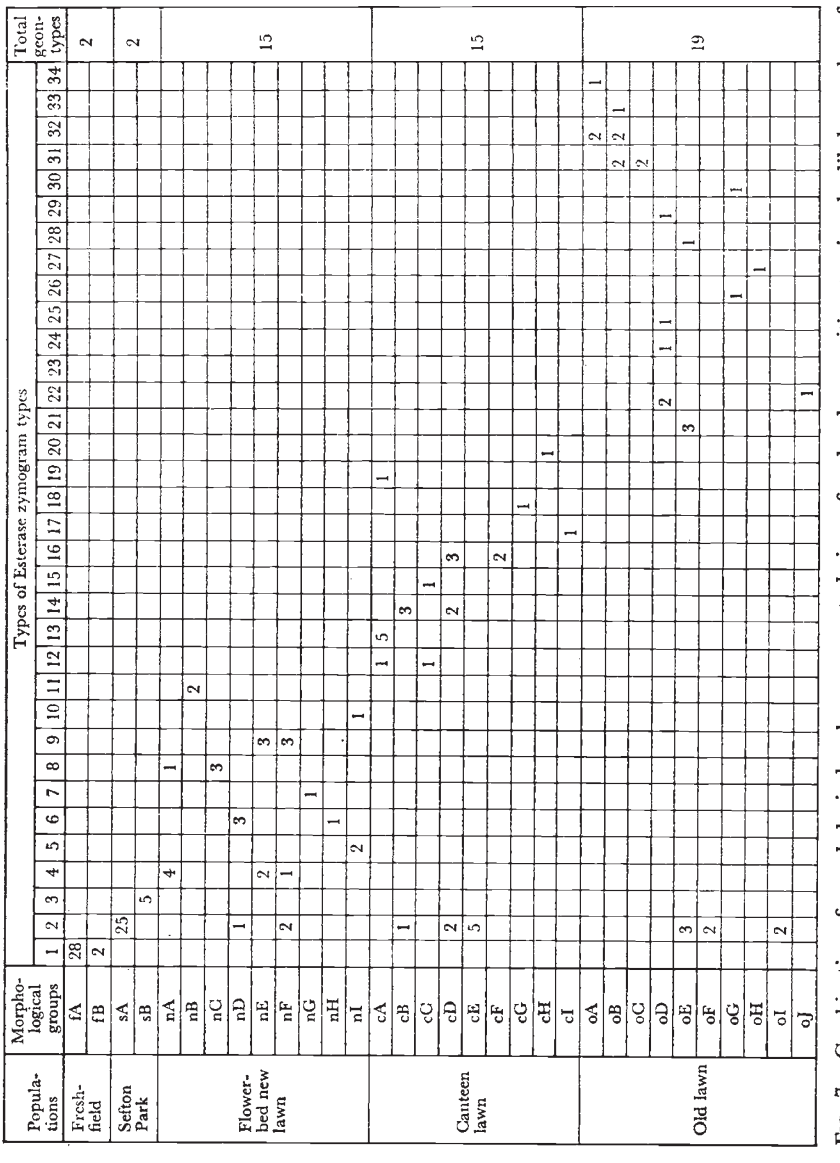

FIG. 7.—Combination of morphological and zymogram techniques for clonal recognition to give the likely number of distinct genotypes in the samples of five populations of *Agrostis stolonifera*.

The three refinery populations are certainly not formed by the spread of a few clones but consist of many different clones. This is borne out in more detail by the analysis of the Canteen lawn population. The Freshfield and Sefton Park populations are in marked contrast. The samples of each appear to be made up of only two clones.

The diversity of genotypes in the older refinery populations can be seen to be a logical consequence of the processes operating in the young refinery populations. A large number of individuals survive through the earlier stages of selection and apparently continue to survive in the later stages. There is no sign of a reduction of number of clones in the oldest refinery population, despite the severity of the environment and continuous mowing.

There is, however, a very low number of clones in two of the uncontaminated populations. This highly simplified population structure is remarkable, and is matched only by populations of *Holcus mollis* (Harberd, 1967). It is difficult to believe that these populations were only started by a few seeds. Reduction of clonal number, perhaps because of long-continued competition, must have occurred. But in one uncontaminated population, from the sea cliff, a great number of clones occurs. It is in a very severe environment in which very few other species can grow and very strong selection pressures operate (Aston and Bradshaw, 1966). It is difficult to know what to conclude, except that conditions of severe selection certainly do not lead to a reduction in the numbers of individual clones in a population, but rather to the maintenance of a large number.

The different individual esterase isoenzymes which occur in the various populations allows us to make some comment on their selective value even although we know nothing of their genetics. There is a great diversity of isoenzymes in the three refinery populations. An analysis (fig. 8) gives no indication that the refinery populations have any isoenzymes particularly in common. This suggests that there is no very strong selection pressure for particular isoenzymes in a copper contaminated environment despite the recent evidence that strong selection pressures can act on esterase isoenzymes in barley (Allard, Kahler and Weir, 1972). But the small number of populations analysed and the simplification of two of the uncontaminated populations make any firm conclusion impossible.

5. Conclusions

The copper refineries at Prescot release considerable quantities of copper into the environment. This copper pollution causes a considerable degradation of the plant communities in the vicinity, so that in the areas of highest pollution only a few species survive. The major species, *A. stolonifera*, has evolved copper tolerance. Field experiments, not reported here, show that the species only survives in the neighbourhood of the factory because its populations are tolerant: normal populations die in a few months in the copper-contaminated soils, tolerant populations survive and grow normally. The experiments with seed material show the same importance of tolerance.

A large number of other species would have been present at Prescot before the pollution began and would have become part of lawns in the absence of copper contamination, but they do not occur there any longer. Several of these species have been shown to lack the genetic variability to evolve tolerance to copper (Gartside and McNeilly, 1974). *A. stolonifera* resembles *A. tenuis* in possessing the necessary variability. Prescot then provides one more example of the ecological amplitude of a species being determined by its genetic amplitude—its capacity to evolve. In the future we will have to pay more attention in evolutionary studies to availability

of variation, for variation, more than selection, appears to be the limiting factor in evolution.

In very many cases of population differentiation in plants we can study only the result of evolution, and we cannot see how fast it occurred, or even how it occurred. The occurrence of a series of lawns of different ages at Prescot provides an ideal opportunity to see that evolution in plants can be exceedingly fast, and that major changes in the characteristics of a population can occur even within a single generation, matching the

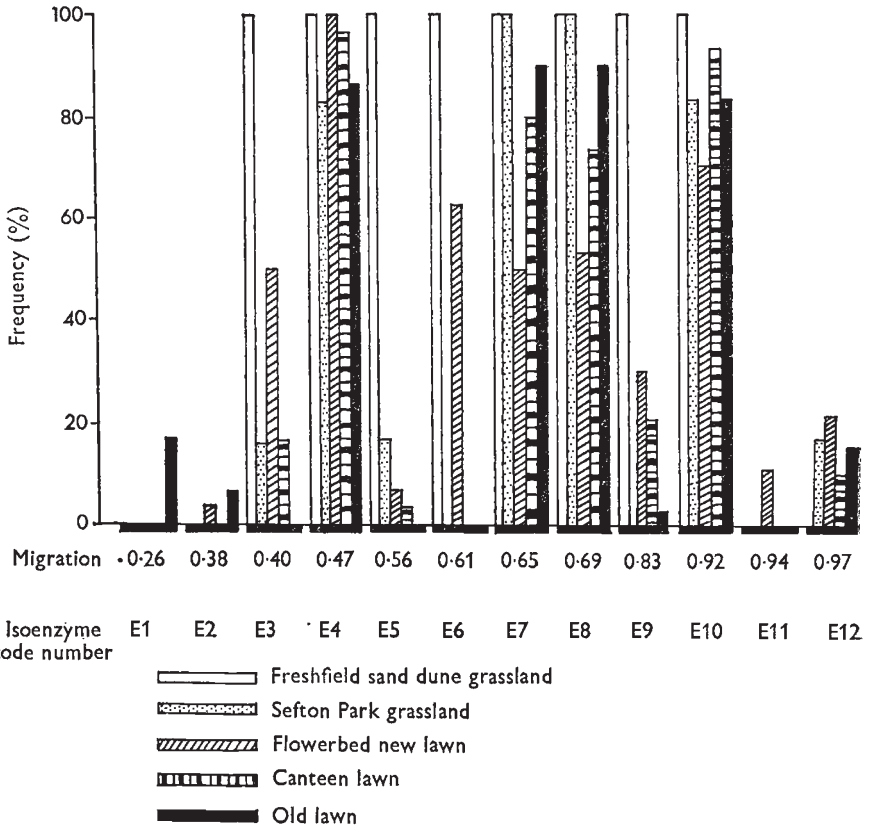

FIG. 8.—The relative frequency of different esterase isoenzymes in the samples of five populations of *Agrostis stolonifera*.

very few other plant examples which are mainly in agricultural situations (Bradshaw, 1972). There is no reason at all why such rapid changes should not occur under conditions of strong selection. Aerial pollution can certainly be a powerful selective force. It has recently been shown to cause changes in other plant species, SO_2 tolerance in the grass *Lolium perenne* (Bell and Clough, 1973), and lead tolerance in the moss *Marchantia polymorpha* (Briggs, 1972): its effects on insects are well known.

The impact of copper contamination uncovers a low frequency of tolerant individuals, which are immediately selected. Amongst these there is a

range of tolerance, and it is not clear whether or not plants with full tolerance occur: they may, at extremely low frequency. The young selected populations are certainly not composed of fully tolerant individuals. These only appear in the old populations, perhaps by recombination, for a small amount of flowering does occur even with mowing.

Despite the very strong selection and the outstanding vegetative reproduction of *A. stolonifera* there is no sign that the populations are at any time composed of only a few genotypes. The clonal analysis suggests that the population size of even the very small lawns (10 m square) has never been less than 100. In these populations then there is no sign that conditions exist which could lead to a founder effect.

The selection acts very strongly at the seedling stage. But since *A. stolonifera* is perennial it can also act on the adult. A relationship can be found between the spread of clones in the contaminated environment of the Flowerbed new lawn and their tolerance. There is no reason why this type of selection should not be extremely important in determining the capacity of an individual to leave descendants, especially since the selection effects can be cumulative in a perennial (Hickey and McNeilly, 1975).

With such strong selection it could be expected that the oldest populations would be uniformly highly tolerant. But it is far from the case: the Old lawn population appears even to be bimodal in its tolerance (fig. 3). In view of the possibility that the distribution of tolerance has been upset by the vegetative spread of individual clones the tolerance data has been replotted (fig. 9) replacing the scores of individuals which appear to be members of the same clone on the evidence in fig. 7 by means for the clone. The considerable variance, and even bimodality, of the Old lawn population, as well as of the other refinery populations, remains.

It is very difficult to provide any explanation for this. The same thing has been found in other metal-tolerant populations (McNeilly and Bradshaw, 1968). In the latter populations there was the possibility that the variability was being maintained by gene flow although it should have been overcome by selection. In the Old lawn population there is no possibility of gene flow. We must look for some selective explanation involving the relative fitness of the individuals under conditions of competition, such as annidation (niche diversity) giving rise to frequency-dependent selection. It is hard to believe that heterozygote advantage plays much part since we are essentially dealing with a non-sexual situation as in barley populations (Allard and Adams, 1969), where variability is strongly retained by frequency-dependent selection.

The existence of genetic diversity in the population is shown not only by the measurements of tolerance but also the evidence on clonal structure. For some reason there are powerful forces retaining variability in this strongly vegetative population just in the same way that there appear to be in clover, *Trifolium repens*, another strongly vegetative perennial (Cahn and Harper, 1975).

But at the same time the complete opposite situation in the two original control populations from uncontaminated environments must not be forgotten and an explanation found. It is interesting that in *Festuca rubra* (Harberd and Owen, 1969) the area where the amount of clonal reduplication was most was in the areas where *F. rubra* was most common, *i.e.* in its optimum habitats. This could be considered to be true in *A. stolonifera*,

suggesting that there is some relationship between the suitability of the habitat and the degree of simplification of the population structure. In *Holcus mollis* the extreme simplification of the populations is related to the existence of sterile chromosome races (Harberd, 1967). But the *A. stolonifera* populations appear to be normally fertile.

The populations at Prescot provide simple evidence for the power of selection and the ease with which some species, but not others, can respond to it. But there are curious attributes of the populations which show that the selective situation has complexities which at the moment we cannot understand.

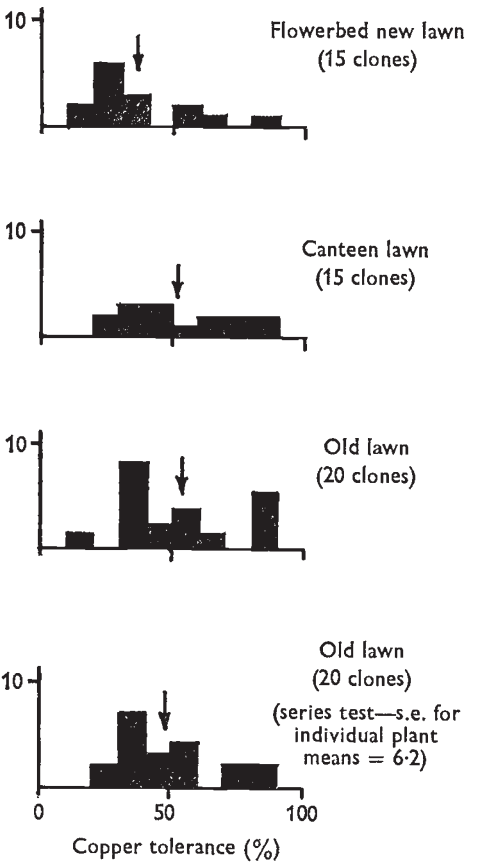

FIG. 9.—Distribution of copper tolerance in the individual clones comprising three refinery populations of *Agrostis stolonifera*.

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