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THE POTENTIAL FOR EVOLUTION OF HEAVY METAL TOLERANCE IN PLANTS

II. COPPER TOLERANCE IN NORMAL POPULATIONS OF DIFFERENT PLANT SPECIES

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SUMMARY

The effects of increase in copper contamination in potting compost/copper mine waste mixtures on the germination and growth of the following species has been examined: *Agrostis tenuis, Poa trivialis, Lolium perenne, Dactylis glomerata, Cynosurus cristatus, Anthoxanthum odoratum, Arrhenatherum elatius, Plantago lanceolata* and *Trifolium repens*. Copper tolerant *A. tenuis* from Parys Mountain was used as control.

Plant dry weight and plant height decreased with increase in proportion of copper contaminated soil in the mixtures, a conspicuous characteristic was the continued survival and growth of some individuals and the death of others at 1-4, 1-8 and mine soil levels.

Agrostis tenuis and *Dactylis glomerata* produced fully copper tolerant survivors at a frequency of 0·08 per cent. By contrast *Poa trivialis, Lolium perenne, Arrhenatherum elatius* and *Cynosurus cristatus* produced survivors having indices of tolerance ranging from 7 to 20 per cent, tolerance, values too low to be considered as fully copper tolerant. *Plantago lanceolata, Anthoxanthum odoratum,* and *Trifolium repens,* failed to produce any survivors at all.

The heritability of copper tolerance in the selected material: *Agrostis tenuis, Lolium perenne, Dactylis glomerata, Arrhenatherum elatius,* and copper tolerant *A. tenuis* was determined.

Heritability is discussed in terms of breeding value. The results suggested that while the evolution of tolerance would be easy in *A. tenuis* it would be difficult in species such as *Lolium perenne.*

The data presented are interpreted as evidence that the exclusion of some species from mine areas is because of their inability to evolve metal tolerance.

1. INTRODUCTION

MUCH of the waste material left behind after the heavy metal mining and smelting enterprises carried out in the past contain levels of metals, *e.g.* lead, zinc and copper, which are highly toxic to many plant species, with the results that the flora of heavy metal contaminated areas is often restricted. Within certain species, metal tolerant ecotypes have evolved, which are able to successfully colonise these areas and such ecotypes have recently been exploited in the reclamation of heavy metal contaminated sites (Smith and Bradshaw, 1970).

The flora of mine sites is often very different from that of adjacent non-contaminated pastures. For example at the disused copper mine at Parys Mountain in Anglesey, North Wales, the dominant vegetation occurring on contaminated soil is *Calluna vulgaris* (L.) Hull, with isolated areas of *Agrostis tenuis* Sibth. In adjacent non-contaminated areas, however, a more diverse flora occurs with typical acid lowland grassland species including *A. tenuis* predominating.

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In the British Isles, the main plant species which colonise heavy metal contaminated environments are as follows: the grasses *Agrostis tenuis* Sibth., *A. stolonifera* L., *Festuca ovina* L., *F. rubra* L., *Deschampsia caespitosa* (L.) Beauv., and *Anthexanthum odoratum* L., and the herbs *Plantago Lanceolata* L., and *Minuartia verna* (L.) Hiern.

Prat (1934), first suggested that the occurrence of plants on heavy metal contaminated soils was the result of natural selection operating on normal populations to produce metal tolerant individuals.

Walley, Khan and Bradshaw (1974) have demonstrated that if *A. tenuis* is screened on soils made up of varying levels of metal contaminated soil and potting compost, tolerant individuals can be selected out at a single step from normal populations. The number of survivors was found to decrease with increase in the proportion of toxic soil in the mixture. This suggested that tolerance is not a universal feature of the species as a whole but is restricted to genotypes within the species, and that the pattern of variation in the species is continuous.

It has been suggested by Bradshaw, McNeilly and Gregory (1965) and Khan (1969) that not all species are able to evolve metal tolerant ecotypes, and are thus not able to colonise contaminated environments. This restriction must be influenced by different edaphic conditions. There is scattered evidence that richer floras occur on calcareous toxic soils than on acid toxic soils (Antonovics, Bradshaw and Turner, 1971), Similarly the flora of normal calcareous soils is generally more diverse than that of normal acid soils. Clearly high base status allows more species to grow, but there is the added effect on toxic soils of both calcium and *p*H in decreasing the solubility, and thus the toxicity, of the heavy metals (Reuther, Smith and Scudder, 1963; Wallace *et al.*, 1966).

The evolution of tolerant populations on toxic areas must, however, ultimately be dependent on the presence of suitable genetic variation within the species as a whole, which, under the appropriate selective forces, would enable the species to become adapted to toxic soil conditions.

This paper examines the distribution of copper tolerance in seed material of eight plant species *not* found growing on copper contaminated areas, and one species which *is* found commonly on copper contaminated soils. In addition the heritability of copper tolerance in the different species was examined as an indication of breeding value.

The soils which make up these toxic sites are not only toxic, but are also low or deficient in essential nutrients such as phosphate, nitrate, potassium and calcium. The species chosen for screening were, therefore, mainly those whose ecology reflected their ability to grow on soils of low nutrient status, so as to minimise any possible effect that soil nutrient status might have in addition to the selective effect of copper.

2. Materials and methods

Seeds of the following plant species, gathered from non-toxic sites and supplied by Messrs Gartons Ltd. of Warrington, and the Welsh Plant Breeding Station, Aberystwyth, were grown on varying levels of copper contaminated soil.

1. *Lolium perenne* L. S23.
2. *Agrostis tenuis* Sibth.

3. *Cynosurus cristatus* L.
4. *Dactylis glomerata* L.
5. *Poa trivialis* L.
6. *Arrhenatherum elatius* (L.) Beauv.
7. *Anthoxanthum odoratum* L.
8. *Plantago lanceolata* L.
9. *Trifolium repens* L.

Copper tolerant *A. tenuis* from Parys Mountain, North Wales, was used as control.

(i) *Preparation of soils*

Soil containing 1850 p.p.m. copper (total) from Parys Mountain copper mine Anglesey, was used as the toxic soil. Mixtures were made up with sterile John Innes No. 2 potting compost in the following proportions:

(ii) *Mixtures*

J.I. Potting compost		Mine soil	Code
alone		—	J.I.
1 part	+	1 part	1-1
1 part	+	2 parts	1-2
1 part	+	4 parts	1-4
1 part	+	8 parts	1-8
—		alone	mine

(iii) *The Selection experiment*

The soil mixtures were placed in seed trays, and a fine uniform surface prepared to ensure that all plants had as far as possible an equal chance of establishment and survival. Watering was from below, the design being such as to avoid contamination by copper in solution from one seed tray to another. The experiment was performed from October 1970 until February 1971 in greenhouse conditions under natural daylength extended by 8 hours using 400 watt mercury vapour discharge lamps giving an approximate 16-hour photoperiod.

Five thousand seeds of each species were sown on a weight/number basis on the surface of the different treatments. The experiment was of a split plot design each treatment being assigned to a whole plot, the species to the sub plots. There were two replicates. Thus 10,000 seeds of each species were subject to selection at each level of contamination.

After 14 days, percentage germination was determined and the results adjusted such that germination in J.I. compost was 100 per cent. After 8 weeks' growth, random co-ordinates were used to locate 10 individuals per replicate which were then clipped to the soil surface, and after drying to constant weight at 80° C. the dry weight of the clippings was measured.

After 16 weeks' growth, two classes of plants could be distinguished, survivors, and dying or dead plants. Dry weight of the clipped green matter produced, and plant height were determined for each class from each species/treatment combination using 10 individuals taken at random from each replicate.

All the survivors from each treatment were transferred to potting compost and grown on in greenhouse conditions. After 3 months, the index of tolerance of 25 plants from each of the J.I., 1-1 and 1-2 mixtures, together with all the surviving material from the 1-4, 1-8 and mine soils, was determined by the method described by McNeilly and Bradshaw (1968) using 0·5 p.p.m. copper solution. Material known to be non-tolerant was tested at the same time as control. It was thought that some of the species used might, after being subject to selection, show a low degree of tolerance not demonstrable at 0·5 p.p.m. copper. Tolerance testing was therefore also carried out at 0·25 p.p.m. copper in solution.

Ten of the selected copper tolerant individuals of the following species were grown over the winter of 1971-72 in a plastic greenhouse: *Agrostis tenuis*, *Lolium perenne*, *Dactylis glomerata*, *Arrhenatherum elatius*, and natural copper tolerant *A. tenuis* from Parys Mountain copper mine.

In the spring of 1972, isolated polycrosses were set up involving two replicates of each of the 10 genotypes. The seed material was collected in August 1972, allowed to mature and sown in normal potting soil. All genotypes were tested for selfing by bagging together three inflorescences just prior to anthesis and examining subsequently for viable seed.

After 16 weeks, 10 randomly selected individuals from each polycross, together with parental material, were tested for copper tolerance by the method previously described.

3. Results and Discussion

(i) *Germination*

It appears that percentage germination (fig. 1) decreases with increasing amount of mine soil in the mixtures in all species except for *Dactylis glomerata* which appears to be unaffected until the 1-8 mixture ($P > 0{\cdot}001$). Previous work by Dilling (1926), has shown that germination of mustard and cress seeds may be delayed by concentrations of leaf salts in excess of 100 p.p.m. At a concentration of 1000 p.p.m., germination was inhibited completely. However, on removal to a lead free culture, the same seeds would germinate. Similarly, Allen and Sheppard (1970), have shown that the germination of *Mimulus guttatus* D.C. is adversely affected by increase in copper contamination.

In contrast, however, McNeilly (1966), has shown that the germination of *A. tenuis* was unaffected when grown on copper contaminated soil from Drws y Coed in Caernarvonshire, North Wales. It is possible that the high organic matter content in this soil leads to the chelation of toxic ions, so that sufficient non-contaminated water may be imbibed by the seed to enable germination to proceed unaffected. Once the plant begins to take up soil water via its roots, toxic copper ions may enter the plant tissues resulting in the death of the majority of plants and the selection of copper tolerant individuals.

It would thus appear that the presence of copper ions adversely affects germination. In this experiment selection occurs at least in part in the initial stages of germination. Apparently therefore selection operates at two stages during the life of the plant. Firstly, only those seeds which possess some degree of tolerance may germinate, and secondly, of those that do germinate, only a small proportion possess sufficient tolerance to survive to maturity.

(ii) *Growth and Selection*

Up to 8 weeks, copper contamination affects the growth of the species examined causing a significant decrease in dry weight accumulation with increase in proportion of copper contaminated soil in the soil mixture (fig. 2). Significant differences ($P > 0{\cdot}001$) in dry weight were found between J.I. soil, 1-1 and 1-2 soil mixtures. However, no significant differences were found between 1-4 and 1-8 soil mixtures and mine soil.

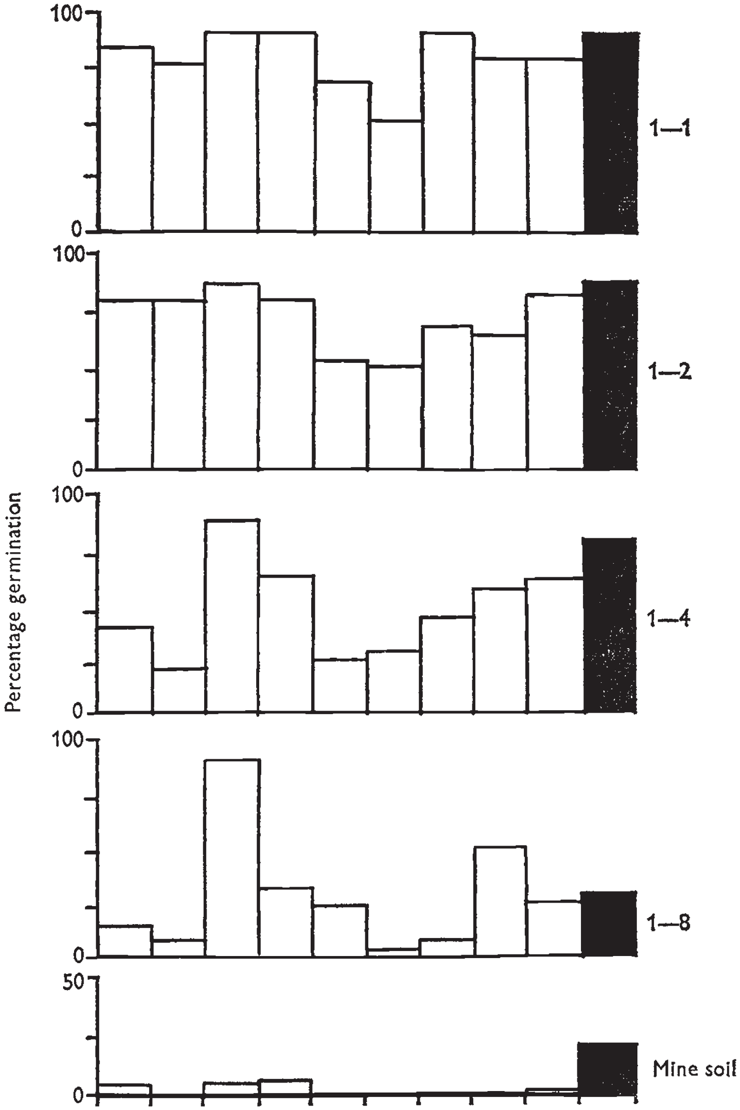

FIG. 1.—Percentage germination of species on varying levels of copper contaminated soil. *Key to species used*: Ae, *Arrhenatherum elatius*; Ao, *Anthoxanthum odoratum*; AtCu, *Agrostis renuis* copper tolerant; At, *Agrostis tenuis*; Cc, *Cynosurus cristatus*; Dg, *Dactylis glomerata*; Lp, *Lolium perenne*; Pt, *Poa trivialis*; Pl, *Plantago lanceolata*; Tr, *Trifolium repens*.

After 16 weeks' growth, two classes of plants could be distinguished in nearly all populations, these being survivors, and dying and dead plants. Significant differences ($P<0{\cdot}001$) between the survivor and non-survivor classes were found for both dry weight accumulation and for plant height (figs. 3 and 4).

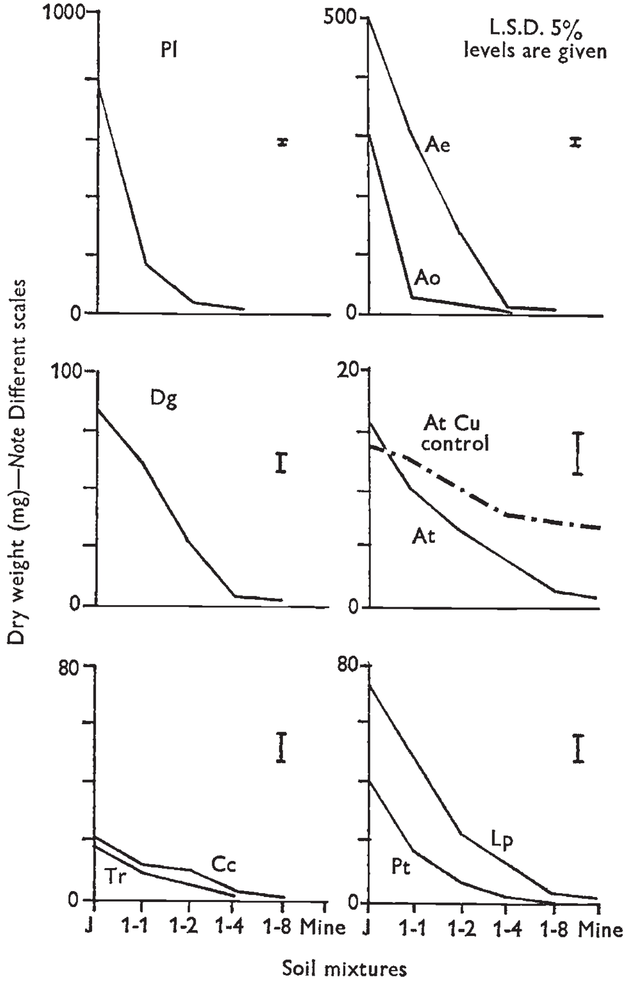

FIG. 2.—Dry weight of different species growing on varying levels of copper contaminated soil. Eight weeks.

The effects of increasing the proportion of copper contaminated soil in the mixture again resulted in significant ($P<0{\cdot}001$) decreases in dry weight and plant height, the survivor class being less affected in all cases.

Survivors could readily be distinguished from dying and dead plants on 1-4, 1-8 and pure mine soils, while at the J.I. 1-1 and 1-2 levels no such distinction could be made. It appears that at the 1-4 level there is an interaction with time so that these two types of individuals become more distinct with the passage of time. It is, however, possible that such a distinction would have been found at the 1-1 and 1-2 levels had the experiment been continued long enough.

Copper tolerant *A. tenuis* showed a decrease in both dry weight and plant height with increase in the proportion of copper contaminated soil in the soil mixture, but the material could not be differentiated into survivors and non-survivors.

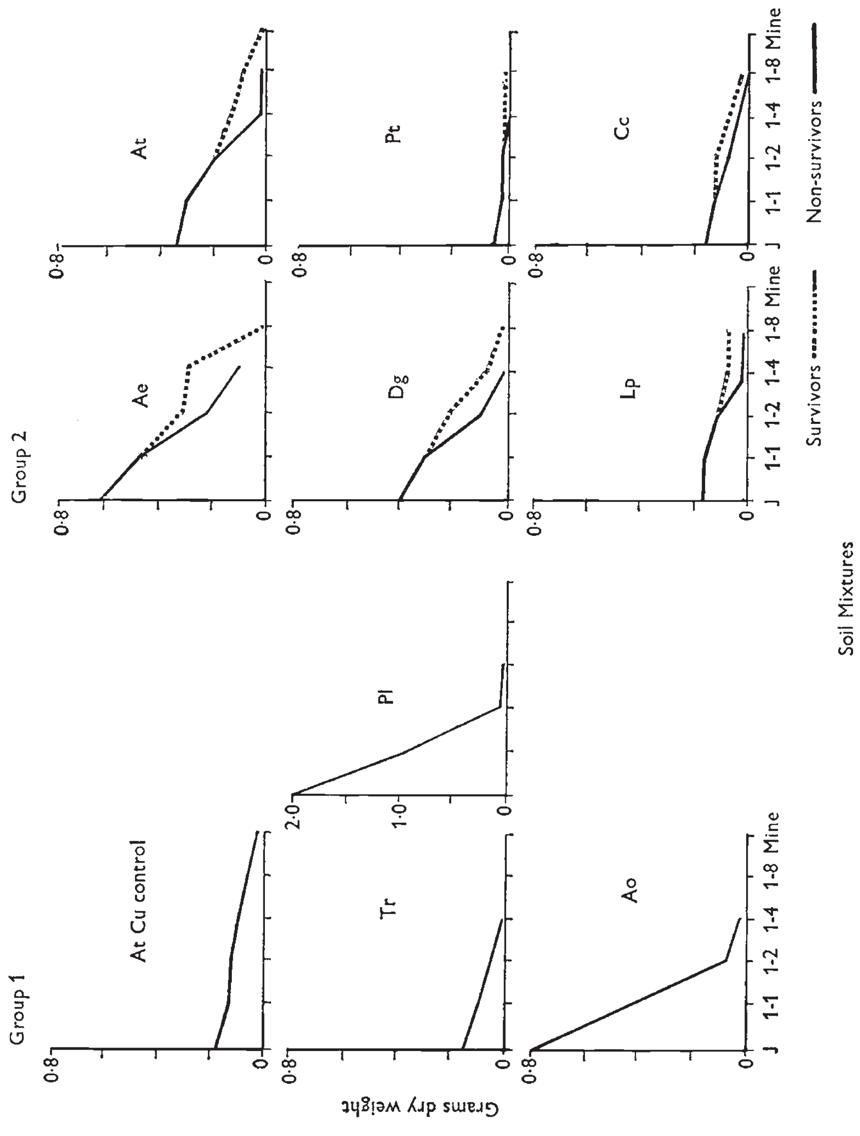

FIG. 3.—Dry weight of different species growing on varying levels of copper contaminated soil. Sixteen weeks.

From this selection response, the species could be divided into two groups.

Group 1. Those incapable of producing any survivors from 10,000 seeds sown on each of the copper contaminated soils: *Anthoxanthum odoratum, Plantago laceolata* and *Trifolium repens.*

Group 2. Those capable of producing some survivors from the 10,000

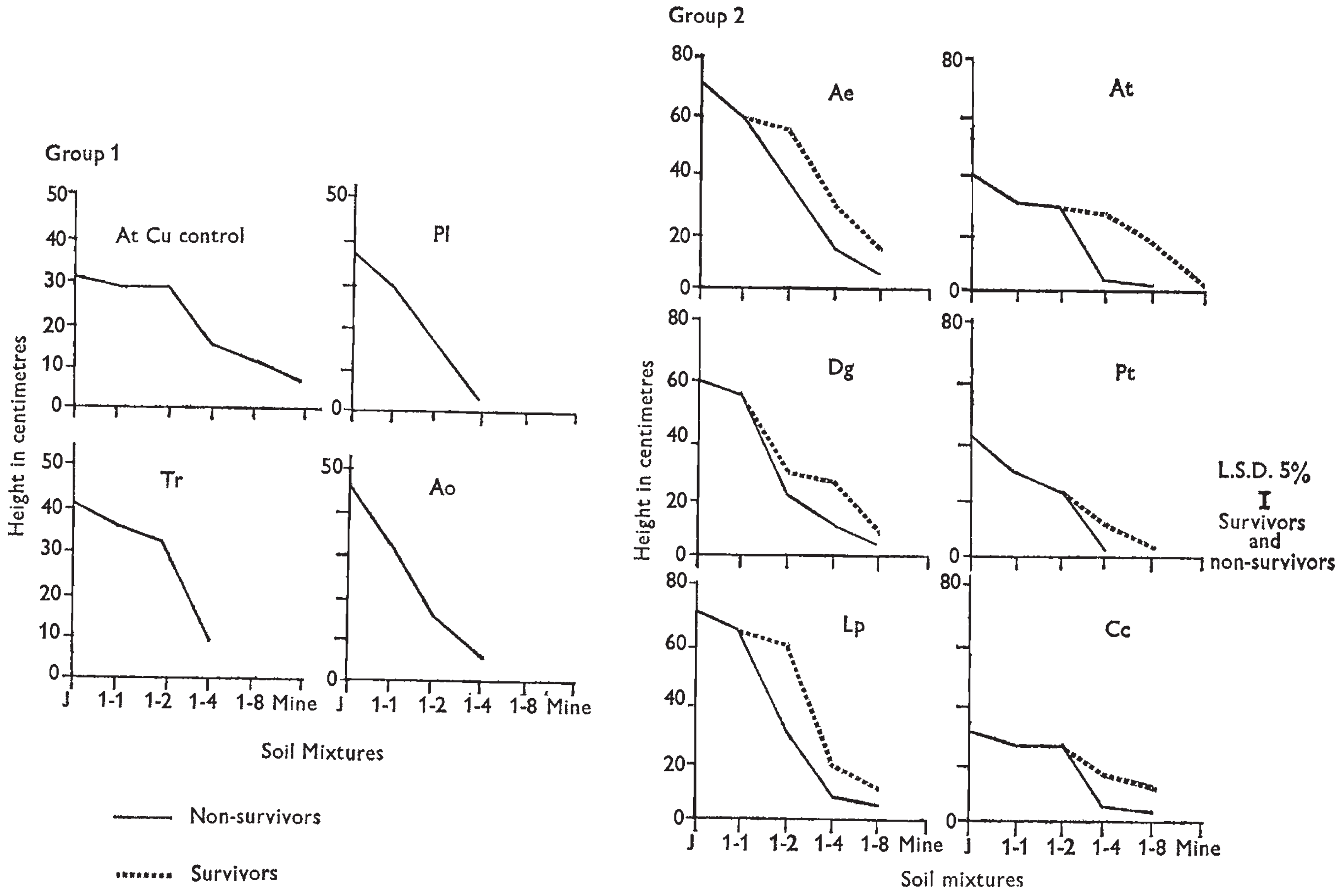

FIG. 4.—Plant height of different species growing on varying levels of copper contaminated soil. Sixteen weeks.

seeds sown on each of the copper contaminated soils: *Agrostis tenuis*, *Dactylis glomerata*, *Poa trivialis*, *Lolium perenne*, *Arrhenatherum elatius*, *Cynosurus cristatus*.

(iii) *Index of tolerance of survivors*

When tested for copper tolerance the survivors from copper tolerant *A. tenuis* control seed grown on mine soil were found to have indices of tolerance ranging from 50 to 95 per cent. Any individual within the survivor class whose index of tolerance was of a comparable magnitude, could therefore be deemed fully copper tolerant.

Individuals of the species comprising group 1, which were taken from the 1-1 and 1-2 levels, failed to produce any roots when tested in either 0·5 or 0·25 p.p.m. copper solutions. Since no survivors were found at levels greater than the 1-2 soil mixture they could not be tested.

Those species comprising group 2, could be subdivided into two groups on the results of testing the index of tolerance of the surviving material (fig. 5).

Group 2A. Species producing survivors having far lower tolerance levels than the copper tolerant *A. tenuis* control but nonetheless showing significant root growth as compared to non-tolerant material tested at the same time. This group comprises: *Lolium perenne*, *Poa trivialis*, *Cynosurus cristatus* and *Arrhenatherum elatius*.

Group 2B. The two remaining species *A. tenuis* and *Dactylis glomerata*, which both produced survivors having full copper tolerance when tested at 0·25 and 0·5 p.p.m. copper respectively.

From the histograms of the index of tolerance of the survivors, it can be seen that increasing the selection for pressure copper tolerance by increasing the proportion of copper contaminated soil in the soil mixtures results in a greater frequency of copper tolerant individuals being detected amongst the survivors. Among the survivors on potting compost alone, 1-1 and 1-2 soil mixtures, the population mean tends towards non-tolerance. On the 1-4 and 1-8 soil mixtures, fully copper tolerant individuals of *Dactylis glomerata* occur. On the 1-4, 1-8, and mine soil alone, fully copper tolerant individuals of *Agrostis tenuis* occur. These individuals were selected from a total of 30,000 seeds of each species sown on these three soils.

Species of Group 2A, follow the same directional trend but at a much lower tolerance level, and never apparently reach full copper tolerance.

(iv) *Heritability*

The polycross material was similarly tested for copper tolerance.

The values for parent/progeny regressions are given in table 1 and are represented graphically in fig. 6. However, since many of the regression coefficients do not appear to differ from one another by more than their standard errors, a single regression coefficient was also calculated using the whole of the artificially selected material irrespective of species, and this value has been used in all further calculations.

The regression coefficient b is equal to half the heritability (*i.e.* $\frac{1}{2}\ h_n^2$) where the following conditions are satisfied. Firstly, the 10 selected genotypes must be a random sample from a randomly mating population in respect of the loci controlling copper tolerance. Secondly, pollination within

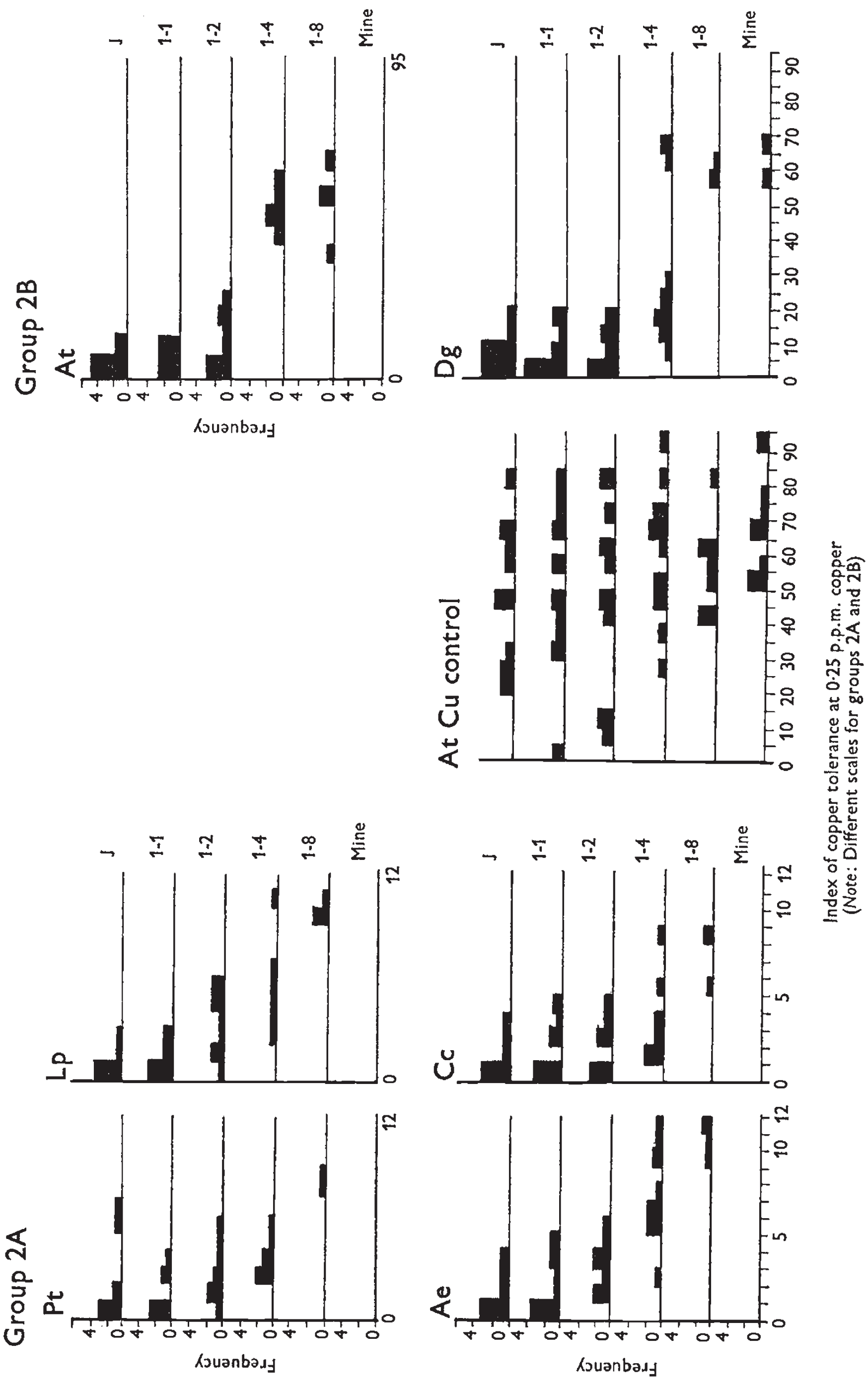

FIG. 5.—Index of copper tolerance of species having survivors on soils of varied copper contamination.

the polycross must be at random. Since in this experiment neither of these conditions is likely to hold, and failure in either would result in a significant bias, the heritabilities, therefore, will lie between *b* and *2b*, according to how closely the conditions are met.

The ready availability of such genetic variation is of interest. Equally interesting is the potential response to further cycles of selection. Such

TABLE 1

Half heritability of copper tolerance (i.e. b $= \frac{1}{2} h_n^2$)

Agrostis tenuis	0·35±0·01
Lolium perenne	0·40±0·07
Arrhenatherum elatius	0·33±0·04
Dactylis glomerata	0·44±0·07
Joint estimate (four species)	0·44±0·026
Copper tolerant *A. tenuis*	0·48±0·20

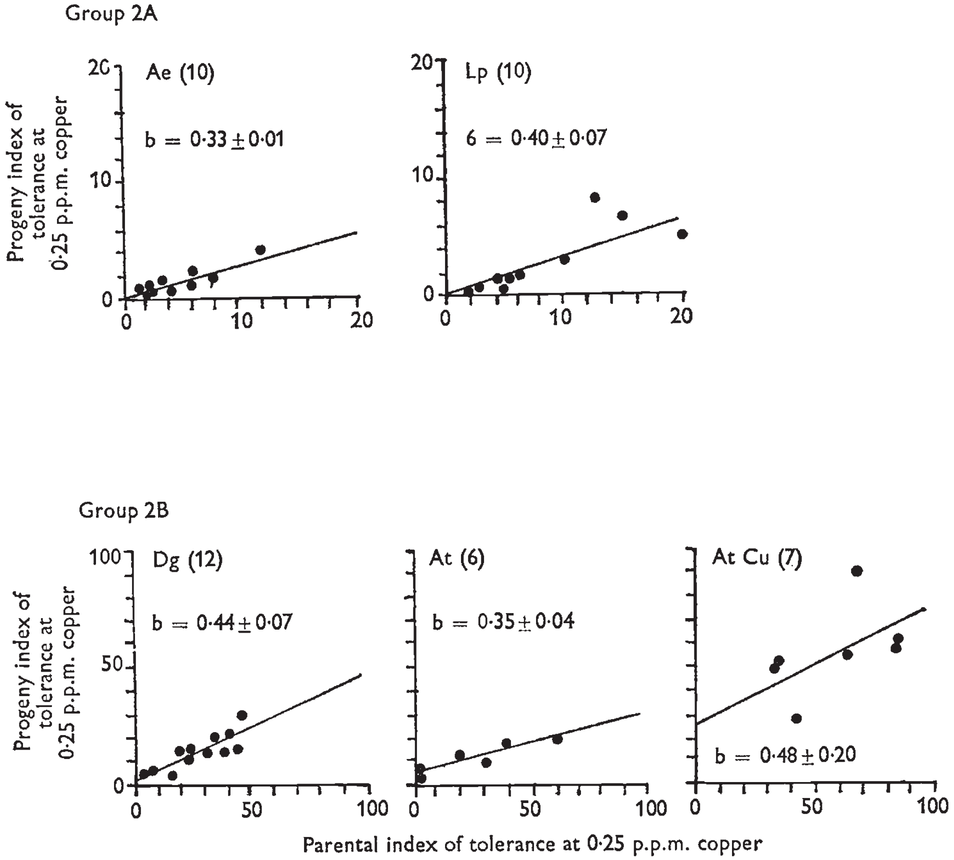

FIG. 6.—Parent/progeny regressions of copper tolerance in different species.

Note: Different scales of tolerance in two groups. Numbers in brackets denote number of individuals per polycross.

information may be derived from the heritability and the selection differential (S), where the selection differential is defined as the mean phenotypic value of the genotypes selected as parents, expressed as a deviation from the population mean, that is from the mean phenotypic value of all individuals before selection was made. The response to selection (R) is equal to the product of the selection differential and the heritability, *i.e.* $R = h_n^2 \times S$, and this value may give an estimate of the increase in copper tolerance with subsequent generations of selection.

Using the mean value of b, calculated from all the artificially selected copper tolerant individuals, as the lower limit of heritability, and the selection differential values given in table 2, it can be seen that in the Group 2A species *Arr. elatius* and *L. perenne*, at least 26, and 14 generations of selection respectively would be required to select fully copper tolerant individuals. However, in the Group 2B species *A. tenuis* and *D. glomerata* six and four generations of selection respectively would be required for the two species to attain full copper tolerance based on the mean value of b and selection differential values given in table 2. This would of course be a function of population size. Nonetheless fully copper tolerant individuals have been produced after just one cycle of selection.

TABLE 2

Selection differential values

Agrostis tenuis	19·50
Lolium perenne	8·40
Arrhenatherum elatius	4·80
Dactylis glomerata	28·00

The inaccuracy of such predictions may be very large because of the inaccuracies in heritability values based on only a few observations. They may also underestimate the number of generations needed, owing to the fact that progressive selection for copper tolerance may result in a decreased amount of additive genetic variance, a greater frequency of homozygous loci for copper tolerance, and thus decrease in heritability of copper tolerance. Leng (1961), has for example failed to show a good correlation between observed and predicted responses to selection in a series of characters in maize, and whilst Clayton, Morris and Robertson (1957) observed that although comparison between observed and predicted responses to selection in *Drosophila melanogaster* were in agreement, certain lines revealed discrepancies between the observed and the predicted responses. These predictions must therefore be viewed with some caution.

4. CONCLUSIONS

The apparent exclusion of *Trifolium repens*, *Anthoxanthum odoratum* and *Plantago lanceolata* from copper contaminated soils may be explained by the lack of genes for copper tolerance within their respective gene pools.

Trifolium repens has never been found to occur on any heavy metal contaminated soils. By contrast *A. odoratum* and *P. lanceolata* are known both to occur on lead/zinc soils, and to have evolved lead/zinc tolerant ecotypes. *A. odoratum* and *P. lanceolata* must therefore possess the necessary genes for lead/zinc tolerance but not for copper tolerance. There is some evidence that this is so (Bradshaw *et al.*, 1969). This indicates separate genetic control processes for lead/zinc and copper tolerance mechanisms and substantiates the evidence for independent tolerance mechanisms reported by Broker (1963) and Gregory and Bradshaw (1965).

Agrostis tenuis and *Dactylis glomerata* are species which can evolve full copper tolerance as a result of one cycle of selection—providing the population is sufficiently large. The frequency of full copper tolerance was found to be 0·8 per cent in the two populations tested. *A. tenuis* is a species which is well known for its ability to evolve copper tolerance and to occur on copper contaminated sites. However, *D. glomerata* is not a species which has so far been found on heavy metal contaminated soils. Nevertheless, it is suggested that given the levels of heritability and selection differential found in this investigation, the evolution of copper tolerant populations of *Dactylis* and their occurrence on copper contaminated areas would appear to be quite feasible. Its apparent exclusion from copper contaminated areas could be due to its inability to tolerate other edaphic factors contributing to the mine environment. This is similar to the findings of Hannon and Bradshaw (1968), who have shown that salt tolerant ecotypes of *A. stolonifera* are as salt tolerant as those of *Festuca rubra*. However, the distribution of *A. stolonifera* in salt marsh environments is limited to the upper marsh areas, whereas *F. rubra* may show a continuous distribution throughout the upper and lower marsh areas. These authors have suggested that other edaphic factors, such as waterlogging, are responsible for the exclusion of *A. stolonifera* from lower salt marsh sites.

Although, as we have seen, *Agrostis tenuis* is capable of producing fully copper tolerant individuals in one cycle of selection, it is also possible that full tolerance may in practice have had its origins from plants such as those selected at sublethal toxicity levels. It has been shown by Antonovics (1968), that the density of *A. tenuis* is greater at the edge of a mine than in the middle, and he argues that the mine/pasture boundary may have lower levels of toxic material owing to the leaching effects of water, and higher levels of nutrients washed off from adjacent agricultural land. He suggests that in such areas, plants having a low index of tolerance may become established and reproduce. Given the levels of heritability and selection differential found for *A. tenuis* in this investigation, fully copper tolerant individuals could then evolve from this large source of individuals in only six generations of selection, although again this will clearly be a function of population size.

The remaining plant species: *Lolium perenne*, *Poa trivialis*, *Cynosurus cristatus* and *Arrhenatherum elatius*, appear to be able to produce individuals with a low level of tolerance in one cycle of selection but never any which are near to full copper tolerance. This may be due to the quality and quantity of genes for tolerance within their respective gene pools. The essential physiological mechanism for a low degree of tolerance must be present but at too low a level to overcome the effect of soil copper.

Given the levels of heritability and selection differential found in this investigation for *L. perenne* and *A. elatius*, it would appear that the restriction of species of Group 2A, must be due to their initial inability to become established even in nursery areas, and also to the low level of extractable genetic variability in copper tolerance.

The data here presented are certainly in agreement with the increasing evidence that many species only have a wide distribution because they have been able to evolve races adapted to particular conditions. If the appropriate genetic variation is not present, this evolution and the consequent widened ecological amplitude cannot occur.

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