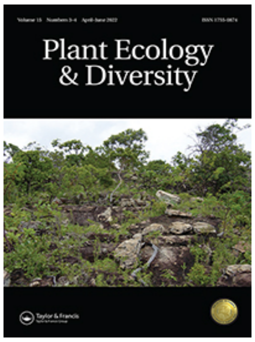

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Edaphic ecotypic divergence in *Senecio vulgaris* and the evolutionary potential of predominantly self-fertilising species

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ABSTRACT

Background: There is continued interest in ecotypic divergence because it is informative of local adaptation and can be an important step in speciation.
Aim: To investigate ecotypic divergence in a predominantly self-fertilising species, *Senecio vulgaris*, in response to variation in soil nitrogen (N) and phosphorus (P) concentrations.
Methods: Responses to increased N and P were compared among populations sampled from sand dune, bird sanctuary and garden habitats, containing soils of low, high and intermediate fertility, respectively. Responses were measured in terms of growth, with capitulum production and days to seed set recorded in some experiments.
Results: Population differences in response to increased N and P were detected and linked to differences in soil fertility among habitats. Thus, the sand dune population exhibited the lowest growth response to increased N and P concentrations, while a bird sanctuary population showed the greatest response to increased N supplied as either $NaNO_3$, NH_4NO_3 or urea. Genetic variation in response to increased N was present in both of these populations.
Conclusions: The results indicate the presence of edaphic ecotypes in *S. vulgaris*, providing further evidence that questions the premise that self-fertilisation limits adaptation to environmental change and thus is an evolutionary dead-end.

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Edaphic ecotypes; local adaptation; nitrogen response; phosphorus response; self-fertilisation; *Senecio vulgaris*; soil fertility

Introduction

Ecotypes are different heritable forms of the same species that are associated with and adapted to different habitats (Turesson 1922, 1925). There has been a resurgence of interest in ecotypic divergence within plant species in recent years because genomic analyses can help reveal what genetic changes occur during local adaptation and ecotype formation (Todesco et al. 2020; VanWallendael et al. 2022; Wright et al. 2022), and also because such divergence can be an important step in speciation (Abbott and Comes 2007; Lowry 2012; Lowry and Gould 2016). Often, different ecotypes are ecogeographically isolated from each other through their adaptation to different habitats such that gene flow between them is much reduced or absent. Under conditions of parapatry, however, differences in flowering time or pollinator specificity can act as effective reproductive barriers, as may other differences reflecting local adaptation to habitats. With regard to the latter, recent studies have shown that very strong selection may act against immigrants due to ecotypes exhibiting highest fitness in their native habitats (Lowry et al. 2008; Richards and Ortiz-Barrientos 2016).

Fundamental to an understanding of ecotypic divergence is determining which traits are involved in adaptation to particular habitats. This is most easily established when habitats differ for one or a few major environmental differences, for example, the presence or absence of toxic levels of heavy metals in soils. High tolerance to these heavy metals (e.g. lead, copper, zinc, iron) allows metal tolerant ecotypes to grow on soils containing toxic levels of such metals, whereas intolerant ecotypes of the same species are unable to do so (McNeilly 1968; Antonovics et al. 1971; Wang et al. 2022). Similarly, adaptations to soils that differ in levels of major nutrients, such as nitrogen (N), phosphorus (P) and potassium (K), have been shown to evolve easily and rapidly in some species (Davies and Snaydon 1974; Davies 1975) and lead to the origin of ecotypes associated with habitats of different nutrient status (Snaydon and Bradshaw 1962; Antonovics et al. 1967; Ramakrishnan and Gupta 1973; Eriksen and Nordal 1989).

However, previous studies linking ecotypic divergence in plants with differences in soil nutrient status have been conducted on outcrossing species, which raises the question as to whether similar divergence has occurred in predominantly self-

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fertilising species. Despite the fact that self-fertilisation can have short-term benefits by providing reproductive assurance to individuals in the absence of pollinators or mates (Darwin 1876; Baker 1955), it is considered to be an evolutionary dead-end due to selfing lineages having a higher risk of extinction over long-term evolutionary timescales relative to outcrossing lineages (Stebbins 1957; Goldberg et al. 2010; Igić and Busch 2013). One disadvantage of self-fertilisation over the long term is assumed to stem from the limited ability of selfers to adapt to changing environments (Takebayshi and Morell 2001) due to selfing causing a reduction in effective population size, which, in turn, reduces levels of genetic variation within populations (Glemin et al. 2006; López-Villalobos and Eckert 2018) on which selection might act. However, selfing promotes population divergence within species (Glemin et al. 2006; Huang et al. 2021) and there is growing evidence that ecotypic divergence occurs readily in predominantly self-fertilising species in response to changes of environment (e.g. Linhart and Baker 1973; Linhart 1974, 1988; Abbott 1976; Neuffer 1990; Volis et al. 2002, 2022; Ågren and Schemske 2012; Anderson et al. 2013; Ågren et al. 2016; Lucek et al. 2019), although evidence for such divergence associated with variation in soil nutrient status remains lacking, despite genetic variation existing for N use efficiency (Loudet et al. 2003).

Here, I examine whether edaphic ecotypes that differ in response to soil fertility exist in the short-lived, annual herb, *Senecio vulgaris* L. (Asteraceae; $2n = 40$), a predominantly self-fertilising species (Campbell and Abbott 1976; Marshall and Abbott 1982) renowned for exhibiting very high phenotypic plasticity in response to different growth conditions (Harper and Ogden 1970). Previous studies have indicated that there are genetic variants within *S. vulgaris* which exhibit elevated levels of lead and salt tolerance, enabling growth on soils containing high levels of these chemicals (Briggs 1976, 1978). In addition, offspring of plants sampled from frequently weeded sites show heritable precocious development relative to those from sites that are not weeded (Kadereit and Briggs 1985; Theaker and Briggs 1993). These findings, along with the rapid evolution of herbicide resistance in the species in response to herbicide applications (Ryan 1970; Holliday and Putwain 1980), suggest that genetic changes have frequently occurred within *S. vulgaris* in response to anthropogenic changes of the environment. However, until now, there have been no reports of the species exhibiting ecotypic diversification with regard to variation in soil nutrient status despite it being found in a range of habitats that differ greatly in this respect (Walter et al. 2020).

The study reported here follows on from a morphometric analysis of differences between four populations of the common non-radiate form of *S. vulgaris* (var. *vulgaris*) sampled from habitats with contrasting soil nutrient status (Abbott 1976). One of these populations, from the sand dunes at Aberffraw, on the southwest side of Anglesey, North Wales, UK (Figure 1), occurs on soils known to contain low levels of N and P (Pemadasa and Lovell 1974), two others are from a bird sanctuary, Puffin Island (Ynys Seiriol), located a short distance off the east coast of Anglesey (Figure 1), where due to the large amounts of excreta deposited by nesting seabirds, soils are expected to be rich in N and P (Ellis 2005; Ellis et al. 2006; Otero et al. 2018), while a fourth population is from a sheltered walled garden in Oxford, England, where the soil likely contains intermediate levels of N and P. When grown from seed on compost in a glasshouse, plants of the sand dune population usually grow to a much smaller size compared with those from the bird sanctuary and garden populations (Figure 1; Abbott 1976). This indicates a lower inherent growth rate of sand dune plants, which is a common feature of ecotypes and species associated with habitats containing soils of low nutrient status (Eriksen and Nordal 1989; Briggs and Walters 1997). Here, I test the hypothesis that growth differences exist between the sand dune and the three other populations of *S. vulgaris* may have evolved in response to differences in soil fertility. I examine whether growth responses to increased levels of N and P vary between populations in a way that might reflect differences in adaptation to the soils of their respective habitats. Because Puffin Island soils are expected to contain high levels of N in a range of different forms following the breakdown of uric acid (the dominant N compound in seabird excrement; ~80%, Otero et al. 2018), population responses to increased N was tested over three different N sources: ammonium nitrate (NH_4NO_3), sodium nitrate ($NaNO_3$) and urea ($CO(NH_2)_2$). Whereas responses to increased N supplied as either NH_4NO_3 or $NaNO_3$ have been examined in previous studies of edaphic ecotypic divergence that included populations from seabird sanctuaries (Antonovics et al. 1967;

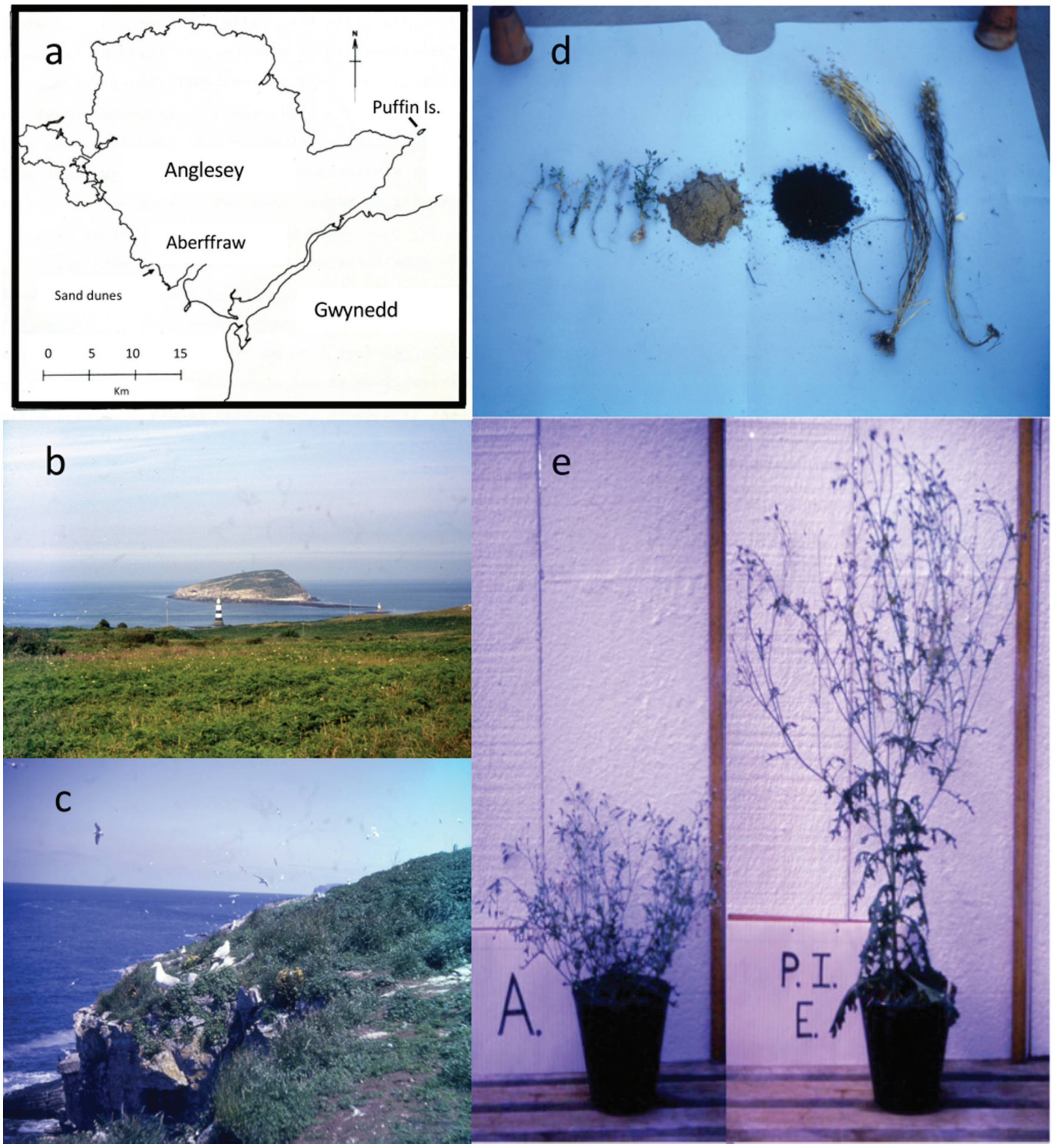

Figure 1. (a) Map showing locations of Puffin Island bird sanctuary and Aberffraw sand dune populations, (b) Puffin Island from Penmon Point, Anglesey, (c) Puffin Island north cliff site, (d) Samples of plants collected from Aberffraw sand dunes (left) and Puffin Island (right), (e) Aberffaw sand dune plant (left) and Puffin Island plant (right) grown from seed on compost in a glasshouse.

Eriksen and Nordal 1989), responses to increased concentrations of urea N have not been tested, despite urea being an early breakdown product of uric acid (Hutchinson 1950), which can be readily absorbed and utilised by plants (e.g. Tan et al. 2000).

Materials and methods

Plant material

Plants in all experiments were raised from seed produced by self-fertilising parent plants grown on compost in heated and illuminated glasshouses at Oxford (see Experiment 3 in Abbott 1976). These parent plants had been raised from seed sampled separately from each of 30 plants per population from populations occurring on the sand dunes at Aberffraw, the bird sanctuary Puffin Island (separately from sites at the north cliffs and central areas of the island), and in a sheltered walled garden at St John's College, Oxford. Descriptions of the locations and sites of populations plus details of sampling are given in Abbott (1976). Hereafter, the Puffin Island north cliff and central populations are referred to as PI north cliff (PINC) and PI central (PICent) populations, respectively. For each population, seed was bulked across the 30 plants parent plants for use in two experiments, but kept separate for use in another experiment (see below).

Nitrogen content of soils

To determine the extent to which soils varied in N content, composite soil samples to a depth of 7.5 cm (after Jackson 1958) were taken from the sites of each population. Throughout the sites of the Aberffraw sand dune, PI central, and Oxford garden populations, five random composite soil samples were collected, whereas at the PI north cliff site five random composite samples were collected from the cliff edge to 5 m inland, and another five from 5 to 10 m inland. Soil samples were collected when *Senecio* populations were sampled for seed and analysed one week later. The nitrate-N content of each soil sample was estimated by means of the phenol-disulphonic acid method conducted after extracting nitrate from 30 g of soil in 150 ml of 0.01 M $CuSO_4$ solution, shaking for 10 min and filtering (Jackson 1958). Exchangeable ammonium-N was extracted by shaking soil samples with acidified NaCl solution (pH 2.5) before quantifying by means of the Kjeldahl still procedure as described by Jackson (1958). N content in the form of nitrate ions (NO_3^-) or ammonium ions (NH_4^+) was expressed as parts of N per million parts of oven dry soil (after Jackson 1958).

Response of populations to increased soil N concentration

Offspring from seed bulked across 30 parent plants derived from each of the Aberffraw sand dune population, both bird-sanctuary populations (PI north cliff and central) and the Oxford garden population were grown to maturity in sand culture over four N concentrations, namely 62.5, 156.25, 312.5 and 562.5 ppm N, with N supplied as either sodium nitrate ($NaNO_3$), ammonium nitrate (NH_4NO_3) or urea ($CO(NH_2)_2$). This range of N concentrations was chosen after a preliminary investigation indicated that population differences in response to increased N supplied as $NaNO_3$ occurred across these concentrations. NH_4NO_3 was used as one source of nitrogen because soil analysis showed that high concentrations of NH_4^+ and NO_3^- were present in Puffin Island soils, particularly those from the north cliff site. $NaNO_3$ was used because it is frequently employed in studies of how plants respond to increased N, and urea was used because it is an early decomposition product of uric acid which is expected to be high in Puffin Island soils. The sand culture technique was similar to the Long Ashton technique (Hewitt 1966) enabling the required range of N concentrations to be established without undue fluctuation during the experimental period. Leighton Buzzard silica sand was used with nutrients removed by immersing sand for one week in 2% HCl followed by repeated washing with tap water and further rinsing with deionised water. Seed was sown on sand contained in pots of 7 cm diameter and 150 ml capacity. Nutrients were applied in the form of a standard nutrient solution adapted from Hewitt (1966) (see Table S1 for composition) with the level of N varied by altering the amount of $NaNO_3$, NH_4NO_3 or urea in the solution. Increasing the N level supplied as $NaNO_3$ inevitably leads to an increase in Na^+ concentration (Table S2). However, no obvious symptoms of Na^+ damage to plants (Tester and Davenport 2003; Flowers et al. 2015) were evident even at the highest concentrations of $NaNO_3$ applied, at which Na^+ concentration was considerably lower than that previously shown to reduce root length and total dry weight of *S. vulgaris* (Briggs 1978). Nutrient solution was applied to cultures (100 ml per application) immediately after sowing and three times per week. To prevent changes in pH and an increase of N concentration from the desired level, cultures were flushed weekly with 200 ml of deionised water after which nutrient solution was applied at double the usual volume.

At the start of the experiment, 25 seeds were sown on the surface of sand in each pot, and a record was taken of germination after 24 days to determine if populations differed in germination across N sources and concentrations. Previous studies of ecotypic divergence had not investigated germination differences between populations in response to soil N, even though such differences could be adaptive with germination divergence being dependent on N source and concentration. After recording germination, seedlings were thinned to three per pot and after 59 days to one per pot. Mature plants were harvested 101 days after sowing with a record taken of shoot dry weight (shoots were dried at 96°C for 36 h before weighing). The experiment was conducted in controlled environmental growth cabinets (Donaldson and Blackman 1973) with temperature maintained at 16°C ±0.5°C and photoperiod set at 16 h during which the intensity of illumination was ca. 305 PPFD (μmol m^{-2} s^{-1}). The experiment was laid out as a randomised complete block design with six blocks, each block representing a different growth cabinet. The total number of plants harvested was 288 representing 6 blocks × 4 populations × 3 N sources × 4 N concentrations.

Data were analysed by analysis of variance (ANOVA) using statistical software distributed by Rothamsted Experimental Station (later developed into the statistical package Genstat). Analysis of germination was conducted following arcsine transformation of percentages, while ANOVAs of dry weight were conducted on both arithmetic and $\log_{10}$ transformed values. Log transformation enables an examination of the growth responses of plants to changes in nutrient level, after eliminating differences due to differences in inherent growth rate (see Antonovics et al. 1967). Thus, if a response difference between populations based on arithmetic data is eliminated after log transformation, it can be concluded that the difference stems from a difference in inherent growth rate. If, however, the response difference is maintained, it may be concluded that it results partly from a difference in inherent growth rate and partly from increased growth due to an additional greater uptake and/or more efficient utilisation of the nutrient supplied at a higher concentration.

Initially, multiway ANOVAs were conducted on the total data set for each character to test for significant differences among main effects (blocks, populations, N sources and N concentrations) and the significance of two-way and three-way interactions between populations, N sources and N concentrations. Subsequently, ANOVAs were conducted separately on data sets for each N source to test for significant differences among blocks, populations and N concentrations, and the significance of the interaction among populations and N concentrations. Regrettably, the original data and full ANOVA tables for this experiment and other sand culture experiments described below were lost during a move of offices. A consequence of this is that standard errors of individual means and mean squares and *F*-values from ANOVAs are not available for presentation.

Genetic variation within populations in response to increased N concentration

It is of interest to determine if genetic variation in response to increased soil N concentration exists within populations as well as between them. To this end, therefore, offspring produced from seed collected and kept separate from each of two mother plants from each of the Aberffraw sand dune and PI north cliff populations were grown to maturity in sand culture over eight N concentrations, namely 9, 22, 65, 174, 261, 348, 521 and 695 ppm N supplied as $NaNO_3$. The two mother plants in each population were chosen to represent extremes of variation in plant size recorded after growth on compost under glasshouse conditions (Abbott 1976). Because *S. vulgaris* reproduces mainly by self-fertilisation and the seed used in the experiment was produced by self-fertilising mother plants (see above), the offspring of these different mother plants can be considered as representing different inbred lines that are genetically distinct from each other. Hence, in the experiment, the offspring of different mother plants are referred to as different genotypes, i.e. A1 and A2 in the Aberffraw population, and P1 and P2 in the PI north cliff population. Seeds were sown on sand contained in pots of 10 cm diameter and 500 ml capacity. Nutrient solution was applied to cultures (300 ml per application) immediately after sowing and three times per week until plants were harvested 13 weeks from sowing when the maximum number of capitula per plant was considered to have been produced. To prevent an increase of N concentration above the desired level, cultures were flushed each week with 600 ml of deionised water after which nutrient solution was applied at double the usual volume. No obvious symptoms of Na^+ damage to plants were evident at even the highest concentrations of $NaNO_3$ applied (Table S2).

Following germination (completed during the first two weeks from sowing) seedlings were thinned to one per pot and at harvest (13 weeks from sowing) number of capitula per plant and shoot dry weight were determined. The experiment was conducted in a glasshouse over a summer period and was laid out along a bench in a randomised complete block design with three blocks. The total number of plants harvested was 96, representing 3 blocks × 2 populations × 2 genotypes per population × 8 N concentrations. Data were analysed by a multiway ANOVA using the same software as above to test first for significant differences and interactions between blocks, populations, genotypes within populations and N concentrations. Subsequently, multiway ANOVAs were conducted on data sets within populations to test for significant differences and interactions between blocks, genotypes and N concentrations.

Response of populations to increased P concentration

Offspring produced from the bulked seed of 30 parent plants per population were grown to maturity in sand culture over six phosphorus concentrations: 0.5, 2.5, 12.5, 62.5, 156.35 and 312.5 ppm P. This range of P levels was chosen because population differences in response to increased P concentration had been detected in other species over a similar range of concentrations (Snaydon and Bradshaw 1962; Davies and Snaydon 1974). The sand culture technique was similar to that described in the first nitrogen response experiment (above) using pots of 7 cm diameter and 150 ml capacity. Phosphorus was varied by increasing the amount of sodium dihydrogen orthophosphate (H_6NaO_6P) in the nutrient solution, which also increases Na^+ concentration but not to levels that harm plant growth (Table S2). A record was taken for each plant of number of days before the first seed was set, after which plants were harvested 13 weeks from sowing and recorded for total dry weight (shoot and root dry weights combined). The experiment was laid out as a randomised complete block design with three blocks in a glasshouse over a summer period. The total number of plants harvested was 72 representing 3 blocks × 4 populations × 6 P concentrations. Data were analysed by a multiway ANOVA using the same software as above to test for significant differences between blocks, populations and P concentrations, and significance of the interaction between populations and P concentrations.

Results

Nitrogen content of soils

Soils from the bird sanctuary PI north cliff and central sites contained considerably more N than those from the garden and dune sites (Table 1). This was evident for both nitrate and ammonium forms of N. However, there was also marked variation of the ammonium-N content within and among PI sites. At the edge of the PI north cliff site where large numbers of seabirds perch and nest, the ammonium-N content was on average twice that of soils from 5 to 10 m inland and between 7 and 8 times that of soils from the PI central site.

Population responses to increased N concentration

ANOVAs of the total data sets for germination and shoot dry weight showed that for both traits the block effect was significant ($P < 0.01$), indicating that the different growth chambers had an effect, but interactions between blocks and other main effects were not significant. Consequently, these interaction terms were combined to produce a residual mean square with 235 degrees of freedom (df) for testing the significance of differences among populations, N sources and N concentrations, and interactions between them.

No significant differences were detected between populations in germination response across different N sources and concentrations (population × N source, population × N concentration and population × N source × N concentration interactions were all non-significant). However, a highly significant N source × N concentration interaction was detected ($P < 0.001$), with germination of populations decreasing from 78% to 50% between 62.5 and 156.25 ppm N supplied as NH_4NO_3, while remaining at 70–80% up to 312. 5 ppm N supplied as either $NaNO_3$ or urea (Figure 2). Significant differences for shoot dry weight (both arithmetic and logarithmic values) were detected between populations, N sources, and N concentrations (each at $P < 0.001$). In addition, two-way interactions were significant between each pair of these factors, while a three-way interaction was significant for logarithmic data ($P < 0.001$).

Table 1. Exchangeable ammonium (NH_4^+) and nitrate (NO_3^-) nitrogen extracted from soils at the Aberffraw, Wales sand dune, Oxford garden and Puffin Island central and north cliff sites. Means with standard errors are provided in parts of N per million parts of oven dry soil (ppm); $n = 5$.

	Nitrogen (ppm)	
	Ammonium – N	Nitrate – N
Sites		
Sand-dune	1.5 ± 0.49	0.33 ± 0.02
Garden	2.7 ± 1.39	0.45 ± 0.04
Puffin Island central	15.3 ± 4.02	17.3 ± 2.09
Puffin Island N cliff		
(a) Edge – 5 m	113.1 ± 6.60	16.1 ± 5.40
(a) 5–10 m	50.1 ± 5.77	19.4 ± 4.28

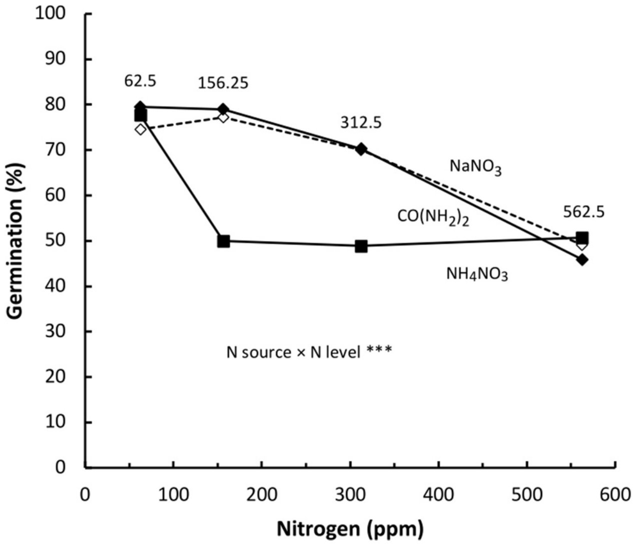

Figure 2. Effect of increasing N level supplied as either NaNO$_3$ (◊), NH$_4$NO$_3$ (■), or CO(NH$_2$)$_2$ (urea, ◆) on mean seed germination pooled across populations of *Senecio vulgaris* from sand dune (Aberffraw, Wales), garden (Oxford) and bird sanctuary (Puffin Island central and north cliff) sites. The significance (***, $P < 0.001$) of the interaction between N source and N level is indicated.

Analyses conducted separately on data for each N source showed that the population × N concentration interaction was significant in all cases for both arithmetic data and logarithmic data with NaNO$_3$ and urea as N sources, and for arithmetic data with NH$_4$NO$_3$ (Figure 3). *Post-hoc* comparisons between population means across N concentrations were made using the least significant difference (LSD at $P < 0.05$) calculated from the residual mean square (Sokal and Rohlf 1981) with 75 df. With N supplied as NaNO$_3$ (Figure 3a,b) both PI populations and the garden population showed similar shoot dry weight responses to increased N, and a markedly greater response than the sand dune population between 62.5 and 156.25 ppm N. The PI north cliff population also produced the greatest shoot weight across all N concentrations indicating its greater inherent growth rate relative to the other populations. With N supplied as NH$_4$NO$_3$ (Figure 3c,d) the PI north cliff population exhibited the greatest shoot dry weight response to increased N between 156.25 and 312.5 ppm and in contrast to the other populations continued to respond positively to increases of N up to 562.5 ppm (although the population × N level interaction was not significant with logarithmic data). With N supplied as urea (Figure 3e,f) the sand dune population showed a negative response to increased N between 156.25 and 312.5 ppm in contrast to the three other populations, which positively responded to this increase in N concentration. Also, above 62.5 ppm the PI north cliff population produced the highest shoot dry weight, indicating its greater inherent growth rate at these concentrations of N supplied as urea.

Genetic variation within populations in response to increased N concentration

ANOVAs of the total data sets for shoot dry weight (arithmetic values) and capitulum number per plant revealed that the block effect and interactions between blocks and the other main effects were not significant. However, differences between populations, N concentrations, the population × N concentration interaction, genotypes within populations, and genotypes within populations × N concentration interaction were all significant ($P < 0.01$-$P < 0.001$) when mean squares were tested against the residual mean square having 62 df. The same was the case for logarithmic shoot dry weight except that the genotypes within populations × N concentration interaction was not significant and the population × N concentration interaction was significant at a reduced level of probability ($P < 0.05$).

ANOVAs conducted separately on data sets of each population also revealed that the block effect was not significant. However, whereas differences between genotypes and N concentrations were significant ($P < 0.01$ – $P < 0.001$) in the PI north cliff population, as was the genotype × N concentration interaction, for both capitulum and shoot dry weight (arithmetic and logarithmic values), in the sand dune population differences between genotypes and the genotype × N concentration interaction were not significant for capitulum number, and

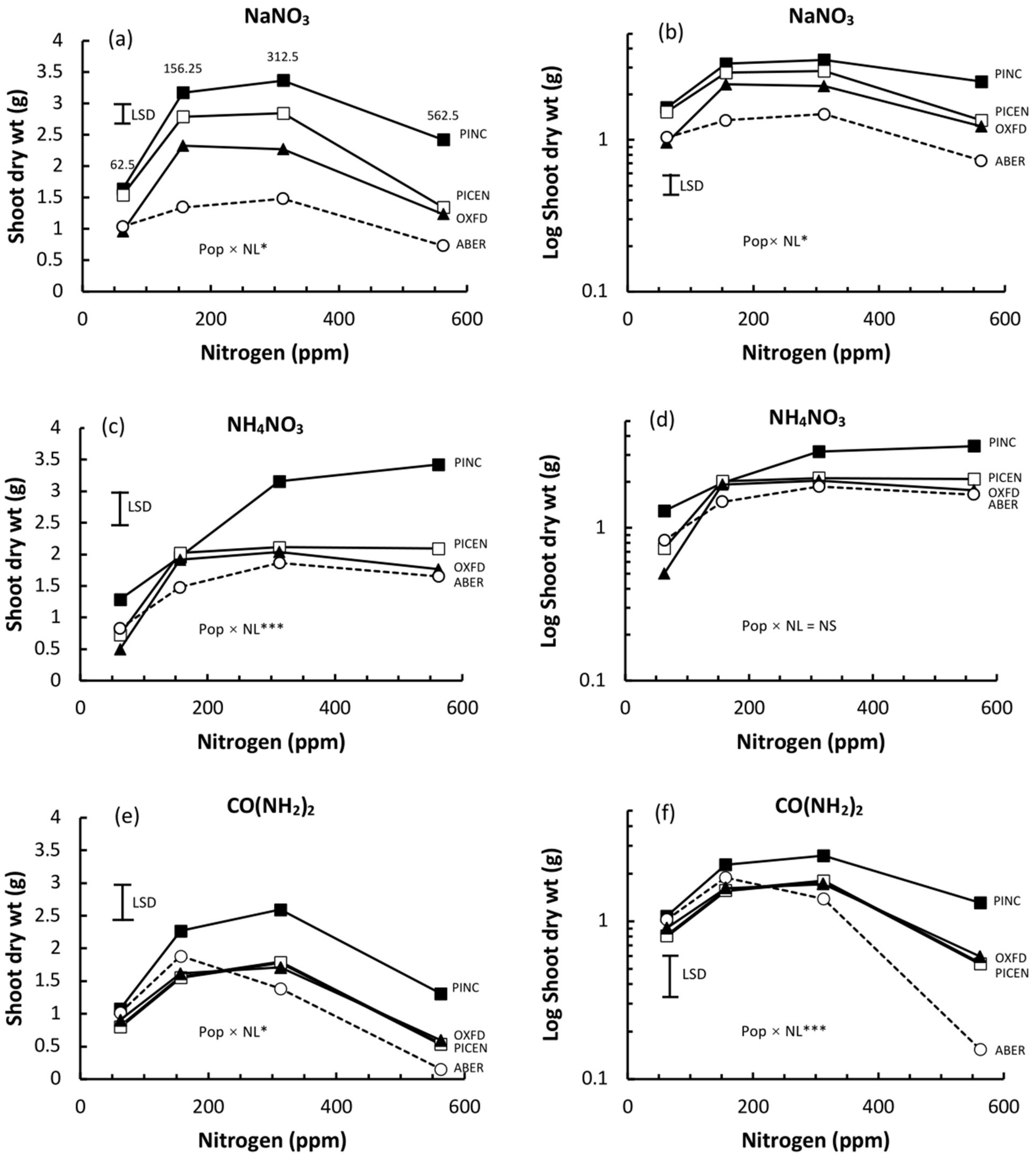

Figure 3. Responses of shoot dry weight of *Senecio vulgaris* to increased nitrogen supplied as $NaNO_3$, NH_4NO_3 or urea ($CO(NH_2)_2$) of Puffin Island north cliff (PINC), PI Central (PICEN), Oxford garden (OXFD) and Aberffraw sand dune (ABER) populations. Plants were grown at 65.5, 156.25, 312.5 and 562.5 ppm N in sand culture as indicated in (a). Pop x NL denotes Population x N level interaction with * and *** indicating significance at 0.05 and 0.001 levels of probability, respectively. Least Significant Differences (LSDs) between means at $P < 0.05$ are presented when the Population x Nitrogen level interaction was significant. Arithmetic means for shoot weight are plotted in a, c and e, while $\log_{10}$ transformed means are plotted in b, d and f.

the interaction term was also not significant for logarithmic shoot weight. The marked differences between genotypes in each population in response to increased N for shoot dry weight (arithmetic values), and for the number of capitula per plant in the PI north cliff population are illustrated in Figures 4a, c,e. In the PI north cliff population one genotype showed a markedly greater response than the other genotype for shoot dry weight production as N increased from 65 to 174 ppm. Similarly, in the sand dune population, one genotype showed a much greater response than the other genotype with the same increase of N and continued to respond to an increase of N up to 261 ppm before its growth was adversely affected at higher concentrations and particularly at the highest N concentration (695 ppm).

Differences between genotype responses were less obvious following $\log_{10}$ transformation of shoot dry weight (Figures 4b,d). Nonetheless,

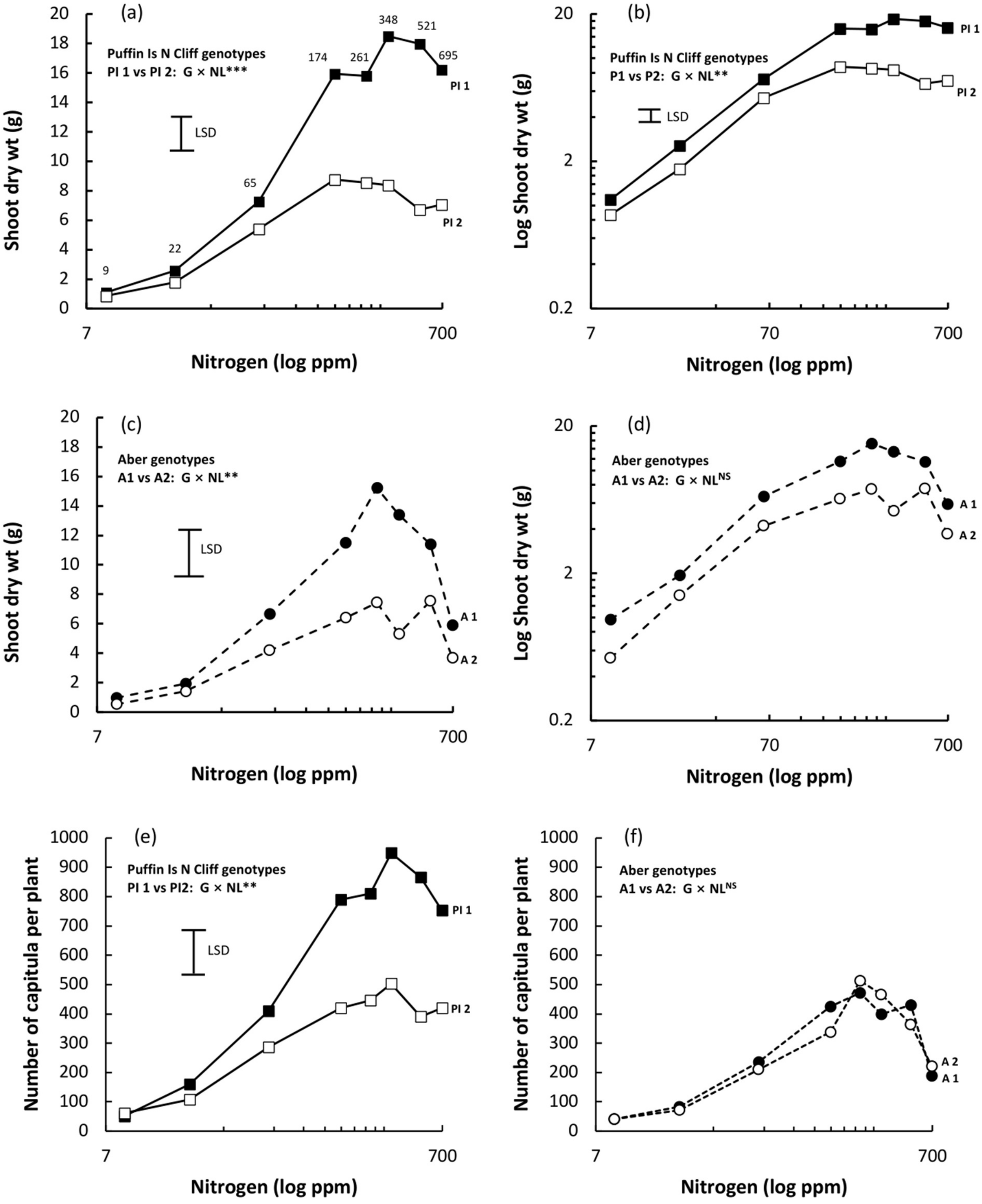

Figure 4. Response of two Puffin Island north cliff genotypes (PI 1 vs PI 2) of *Senecio vulgaris* and two Aberffraw sand dune genotypes (A1 vs A2) to increased N supplied as $NaNO_3$: (a,c) shoot dry weight, (b,d) $\log_{10}$ shoot dry weight, (e,f) number of capitula per plant. G x NL denotes genotype x N level interaction within each population analysed separately, with * and *** indicating significance at 0.05 and 0.001 levels of probability, and NS not significant. Least Significant Difference (LSD) between means at $P < 0.05$ is shown where G x NL is significant. Plants were grown at 9, 22, 65, 174, 261, 348, 521 and 695 ppm N in sand culture as indicated in (a).

the highly responsive PI north cliff genotype maintained its greater response between 65 and 174 ppm N, and produced very large plants at the highest N concentration (Figure 4b). This indicates that its greater growth response was partly due to a higher inherent growth rate and partly to additional physiological differences possibly involving greater uptake and more efficient utilisation of N. Genotype differences in capitulum number response (Figures 4e,f) largely reflected those evident for shoot dry weight, except that both sand dune genotypes exhibited similar responses for this trait. Over all N treatments, the capitulum number per plant was highly correlated with shoot weight ($r = 0.93$, $P < 0.001$ for all four genotypes; $r = 0.99$, $P < 0.001$ for both PI north cliff genotypes; $r = 0.82$, $P < 0.001$ for both sand dune genotypes).

Population responses to P concentration

ANOVAs of the total data set for time to first seed set and total dry weight (arithmetic and logarithmic values) showed that block differences and interactions between blocks and other main effects were not significant. In contrast, differences between populations, P concentrations and the interaction between these two main effects were highly significant ($P < 0.001$) when mean squares were tested against the residual mean square having 46 df. Plants of the PI north cliff population took longer to set seed than those of the other populations, particularly at the lowest (0.5 to 12.5 ppm) and highest (312.5 ppm) P concentrations (Figure 5a). Time to seed set was greatest in all four populations at the lowest P concentration (0.5 ppm). For total dry weight, both PI populations showed a greater response (arithmetic values) to increased P concentration between 0.5 and 2.5 ppm relative to the sand dune and garden populations (Figure 5b). Also, both PI populations showed a markedly greater response than the sand dune

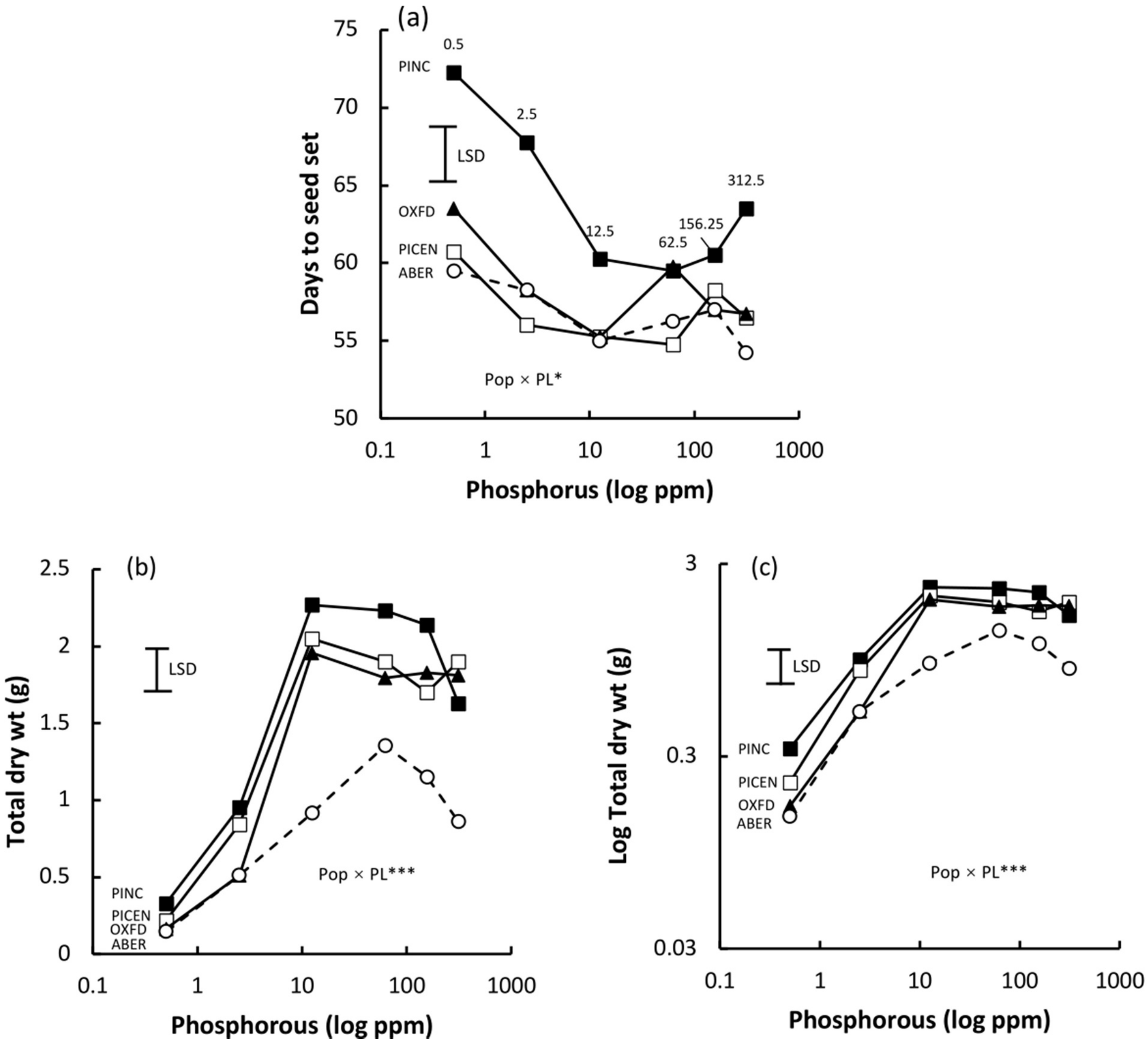

Figure 5. Response of Puffin Island north cliff (PINC), PI Central (PICEN), Oxford Garden (OXFD) and Aberffraw sand dune (ABER) populations of Senecio vulgaris to increased P concentration. (a) days to seed set, (b) total dry weight, (c) $\log_{10}$ total dry weight. Pop x PL denotes Population x Phosphorus level interaction with * and *** indicating significance at 0.05 and 0.001 levels of probability. Least Significant Difference (LSD) between means at $P < 0.05$ is shown where P x PL is significant. Plants were grown at 0.5, 2.5, 12.5, 62.5, 56.25 and 312.5 ppm P in sand culture as indicated in (a).

population, but not the garden population, with a further increase of P up to 12.5 ppm. However, whereas dry weight production plateaued in the PI and garden populations at 12.5 ppm P and above, it continued to rise in the sand dune population up to 62.5 ppm P, before declining at higher P concentrations. At all P concentrations apart from the highest, plants of the PI north cliff population were largest, as reflected in their greater mean dry weight.

Examination of shoot dry weight response on a logarithmic scale (Figure 5c) indicated that the difference between the PI and sand dune populations as P increased from 0.5 to 12.5 ppm was largely the result of the greater inherent growth rates of the PI populations. However, the greater response of the garden relative to the sand dune population between 2.5 and 12.5 ppm P appeared to be due to additional physiological differences other than a difference in inherent growth rate as both populations produced very similar weights at 2.5 ppm P.

Discussion

The analysis of four populations of *Senecio vulgaris* sampled from habitats differing in soil fertility showed that plants from a sand dune population, which occurs on soils of very low fertility (Pemadasa and Lovell 1974), exhibited a lower growth response to increased N and P concentrations applied in sand culture than populations from a bird sanctuary (Puffin Island) and a garden. Growth was measured in terms of dry weight production, which was highly correlated with flowering head (capitulum) number when both traits were recorded. It was also evident that one of the PI bird sanctuary populations (the PI north cliff population), which occurred on soils that were especially rich in N, showed the greatest growth response to increased N supplied as either $NaNO_3$, NH_4NO_3 or urea. From these results, it is concluded that edaphic ecotypic variation related to soil nutrient status is present in *S. vulgaris*.

Differences in soil N content and responses to increased N concentration

Soil analyses showed that there were large differences in nitrate and ammonium N contents between soils on which the four populations occur. As expected, sand dune soil had the lowest N content, Puffin Island soil had the highest, while the garden soil had a low content, though not as low as that of sand dune soil (Table 1). Interestingly, considerable variation in ammonium N level was detected between soils from the two PI sites and also within the PI north cliff site, with levels declining markedly from the cliff edge, where large numbers of seabirds nest, to the central site where a dense mixed stand of *Smyrnium olusatrum* (Alexanders) and *Urtica dioica* (Stinging nettle) occurred and seabirds were less common. Ellis et al. (2006) have noted similar marked differences in soil ammonium and nitrate N contents occurring over short distances on other bird sanctuary islands and related these to the density of nests of gulls and cormorants, while Sobey and Kenworthy (1979) have related such changes within herring gull colonies (in eastern Scotland) to the spatial distribution of defaecation sites. In both of these studies, soils were also reported to contain high levels of P. Caution is required in accepting the accuracy of estimated N contents in the present study as they are based on analyses conducted one week after soils were sampled from the field, during which time levels of N, particularly ammonium N, would be expected to fall due to nitrification (Jackson 1958). Nonetheless, the estimates provide a guide to the relative nitrate and ammonium N contents of these soils. Though not quantified here, urea is also expected to occur at high concentration in PI soils, particularly at the PI north cliff site, due to it being an early breakdown product of uric acid, of which bird excrement is largely composed (Hutchinson ; Otero et al. 2018).

In sand culture, there was no indication that seeds of the PI north cliff population germinated better than seeds of the other populations at higher concentrations of N supplied either as NH_4NO_3, $NaNO_3$ or $CO(NH_2)_2$. However, the PI north cliff population exhibited greater growth (shoot dry weight) at these higher concentrations of N and a greater growth response to increases in N. The latter was particularly marked with N supplied as NH_4NO_3. In contrast, the sand dune population tended to exhibit the lowest growth at higher concentrations of N. The greater growth response of the PI north cliff population, relative to those of the other populations, and similarly the often lower growth response of the sand dune population, were partly due to differences in inherent growth rate between populations. However, because some differences in response were maintained after logarithmic transformation of weights, these were also likely due, in part, to additional physiological differences between populations, such as differences in uptake and efficiency of utilisation of N at higher concentrations of the element (see Antonovics et al. 1967).

In addition to differences between populations, it was evident that genetic variation existed in both the PI north cliff and sand dune populations for growth response to increased N supplied as $NaNO_3$, although only two genotypes were investigated per population. In both of these populations, one genotype exhibited a much greater growth response than the other genotype it was compared with. In the PI north cliff population this difference between genotypes was mirrored by a comparable difference in capitulum number response, but in the sand dune population both genotypes showed equivalent responses for capitulum number. It is difficult to provide reasons for why the two sand dune genotypes did not exhibit comparable differences for both growth and capitulum number responses, although this finding does suggest that genetic variation exists in this population for how shoot dry weight is partitioned amongst vegetative and reproductive structures of the shoot across a range of N levels as previously indicated within *Arabidopsis thaliana* (de Jong et al. 2019). Previous studies of population differences in response to increased N concentration (Antonovics et al. 1967; Ramakrishnan and Gupta 1973; Eriksen and Nordal 1989) did not examine genetic variation within populations, although such variation could potentially aid offspring produced by a population to establish on soils of high or low N status, which might be of particular benefit to a colonising species like *S. vulgaris* where population turnover is likely to be high. How such variation is maintained within populations can only be speculated on. The environments of both the sand dune and PI north cliff populations are spatially heterogeneous in terms of degree of exposure (Abbott 1976), while the habitat of the PI north cliff population is also spatially heterogeneous for soil N content (Table 1). It would be of interest to determine if selection might favour particular genetic variants in different microenvironments (i.e. multiple-niche selection, Levins 1968; Brisson 2018; see also Schemske 1984) and result in the maintenance of genetic variation in growth rate and growth response to increased N in these two populations.

Population differences in response to increased P concentration

Differences in P content between soils were not estimated in the present study but are likely to reflect those for N content, as bird sanctuary/colony soils are known to contain high levels of both nutrients (Otero et al. 2018), while Aberffraw sand dune soil was previously reported to contain low levels of both elements (Pemadasa and Lovell 1974). Population differences in growth and response to increased P concentration were similar in kind to those recorded for increased N level with sand dune plants showing the lowest response. It was also notable that PI north cliff plants exhibited a longer period of growth before seed set at most P concentrations, which might enable them to grow to a larger size and increase their reproductive output, although not when nutrients are limiting.

Growth response, nutrient uptake and reproductive output

Several previous studies of plant ecotypic divergence in response to variation in soil nutrient content have used sand culture or other methods to examine differences in responses to increased concentrations of N and P. These studies showed that populations occurring on nutrient-rich soils often exhibit greater responses to added nutrients, for example, in dry weight production, relative to those from nutrient-poor ones (Snaydon and Bradshaw 1962; Antonovics et al. 1967; Ramakrishnan and Gupta 1973; Eriksen and Nordal 1989). Of particular relevance to the present study are reports that plants of the grass *Lolium perenne* from Puffin Island exhibited a greater growth response to increased N concentration supplied as $NaNO_3$ relative to plants from sites of low soil fertility (Antonovics et al. 1967), and that offspring of a population of *Cochleraria officinalis* from a 'bird cliff' site in northern Norway produced greater dry weight at the highest concentration of N supplied as NH_4NO_3 (Eriksen and Nordal 1989). Moreover, with regard to added P, Davies and Snaydon (1974) have reported that response differences in the grass, *Anthoxanthum odoratum*, can evolve rapidly and over short distances among populations occurring on soils differing in P concentration.

Interestingly, in the study of *C. officinalis*, N uptake was greatest in the 'bird cliff' population at the highest concentration of applied soil N, and lowest at all concentrations of applied N in a population originating from a low N soil. In contrast, Snaydon and Bradshaw (1962) have found that P uptake was greater by populations of *Trifolium perenne* from low P soils than from high P soils, at all levels of P applied in sand culture. These results indicate that edaphic ecotypes may differ in their uptake of an element applied at different concentrations, depending on the nutrient concerned (N or P) or the species investigated. Nitrogen and P uptake and efficiency of utilisation by plants was not investigated in the present study but would clearly be worth examining in the future based on the results of these previous investigations and our current understanding of the mechanisms involved in the processes (Masclaux-Daubresse et al. 2010; Decouard et al. 2022).

None of the previous studies that investigated ecotypic divergence with respect to N and P soil content examined whether differences in growth might be correlated with differences in reproductive output. However, this was indicated in the present study by a very high correlation between dry weight and capitulum production per plant in one experiment where both traits were recorded. An ability to grow to a large size at high N and P concentrations will result in a high reproductive capacity and help provide a fitness advantage over plants of smaller size. In contrast, at low soil N and P concentrations, such as those found in sand dune soils, a small plant size and lower growth response to increased N and P concentrations may be advantageous. This is because small plants with low growth rates will make low demands on soil nutrient reserves, enabling increased survivorship and reproductive capacity when soil nutrients are scarce (Eriksen and Nordal 1989). However, this might not be the case in all species. For example, in the study of *T. repens* by Snaydon and Bradshaw (1962), one population originating from a low P soil produced larger plants at low, intermediate and high concentrations of applied P in sand culture than populations from high P soils. This contrasts with what was found for *S. vulgaris* in the present study and more generally with findings from other studies.

Evolutionary potential of self-fertilising species

All previous studies that demonstrated ecotypic differences in N and P response were conducted on outcrossing species, rather than a predominantly self-fertilising species, as was the case here. The findings reported here add to the growing evidence that ecotypic divergence frequently occurs in predominantly self-fertilising species in response to differences of environment (e.g. Linhart and Baker 1973; Linhart 1974, 1988; Abbott 1976; Neuffer 1990; Volis et al. 2002, 2022; Ågren and Schemske 2012; Anderson et al. 2013; Ågren et al. 2016; Lucek et al. 2019) and that such species (e.g. *Arabidopsis thaliana*) contain variants for a wide range of ecologically important traits (e.g. Weigel 2012; Vasseur et al. 2018). Such selfing species, therefore, do not appear to have a limited ability relative to outcrossing species to adapt to changing environments, at least in the short term. Nonetheless, self-fertilisation is considered to be an evolutionary dead-end (Stebbins 1957; Igić and Busch 2013) showing a higher rate of extinction over long-term evolutionary timescales relative to outcrossing lineages (Goldberg et al. 2010). The disadvantage of self-fertilisation over the long term has been assumed to stem from a limited ability to adapt to changing environments and/or susceptibility to the accumulation of deleterious mutations (Takebayshi and Morell 2001). Clearly, the growing evidence of ecotypic divergence within predominantly selfing species calls into question the first of these reasons. Moreover, deleterious mutations are expected to be purged from self-fertilising lineages (Crnokrak and Barrett 2002; but see Cheptou 2019), such that long-term self-fertilising species should not suffer from the effects of inbreeding depression due to expression of deleterious recessive alleles.

The presence of a range of different ecotypes adapted to different environments within a species may provide preadaptation to future environmental change, should such change be already encountered and adapted to in part of a species range (Crawford 2008). Consequently, ecotypic divergence could enable a species to adapt to environmental change over the long term as well as the short term. It is difficult, therefore, to accept that an inability to adapt to environmental change is a major cause of the higher risk of extinction of self-fertilising species, relative to outcrossing lineages, over long-term evolutionary timescales. Clearly, more studies conducted across a large number of self-fertilising species are required to test this hypothesis further.

An important note of caution in interpreting the results of the present study, and similar studies of population divergence within species, concerns whether such divergence is actually adaptive. Reciprocal transplantation experiments that test the fitness of plants in their own habitat against those of other conspecific populations remain the best way of testing adaptive divergence, and in only a few studies of self-fertilising species have such experiments been conducted (Schemske 1984; Volis et al. 2002; Ågren and Schemske 2012; Anderson et al. 2013; Ågren et al. 2016). Associations between particular genotypes and habitats can be misleading, with transplantation experiments showing that there may be no adaptive basis for them (Latta 2009). Although physiological differences between populations provide more compelling evidence for adaptive divergence, transplantation experiments are required to prove this.

Conclusions

The results reported here show that population divergence in response to variation in soil fertility and N and P concentrations has occurred in *S. vulgaris*, with differences between populations related to levels of N and P in the soils they occur on. The different populations can be considered to represent different ecotypes of the species, although transplant experiments are required to confirm this. In addition to population differences, genetic variation in response to increased N concentration was detected within both a sand dune population and a bird-sanctuary population (Puffin Island north cliff). Thus, standing genetic variation exists within *S. vulgaris* in terms of growth response to major soil nutrients both between and within populations. This and related evidence from other predominantly self-fertilising species, most notably *A. thaliana*, suggests that such species are often likely to contain genetic variation for a wide range of ecologically important traits, thus providing the potential for evolutionary change over both short and long term timescales.

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