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Performance of four *Dactylorhiza* species over a complex trophic gradient

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SUMMARY

Spontananeous distribution and survival in experimental plots of four marsh orchids (*Dactylorhiza* spp.) in a hay-meadow complex were related to mineral composition of groundwater, soil nutrient availability and species composition of the vegetation. Differences in Ca^{2+} contents of the groundwater pointed to the discharge of various groundwater flows in the meadow. Soil fertility and extent of N limitation, measured with the phytometer *Cirsium palustre*, varied with position on the gradient. Total N content of the soil and levels of exchangable K^+ and NH_4^+ were correlated to organic matter content. Highest total P levels were found in areas with a pronounced discharge of mineral-rich groundwater. Concentration of available P was higher in wet than in drier sites. Natural abundance and survival of introduced *Dactylorhiza maculata* (L.) Soó plantlets was highest in *Juncus acutiflorus*-dominated transitions between meadows and *Parvocaricetea* marsh. These were characterized by the occurrence of a water type of intermediate mineral content and by low soil fertility, limited by N and P. Survival of *D. praetermissa* (Druce) Soó showed exactly the opposite pattern to that of *D. maculata.* It had its optimal spontaneous abundance in more fertile parts of the meadow influenced by discharge of groundwater with an intermediate mineral content. *D. majalis* (Rchb. f.) Hunt & Summerh. also occurred in the more fertile parts of the meadow, but showed an optimum in drier stands associated with discharge of highly calcareous groundwater. The phosphorus-pool of these soils was high, but the availability of phosphate was low. The soil fertility of *D. majalis* site was limited by N. *D. incarnata* (L.) Soó occurred mainly in transitions between basiphilous small sedge vegetation and productive hay meadow stands. The species occurred at fertile but waterlogged soils with high concentrations of NH_4^+. The presence of base-rich groundwater with a high pH apparently prevents the species from ammonium toxicity. None of the species occurred in degenerated (drained) and acidified meadow sites.

Key-words: bioassay, *Calthion palustris*, *Dactylorhiza* spp., groundwater composition, nutrient availability, vegetation composition.

Nomenclature: Van der Meijden *et al.* (1990) for vascular plants; Schaminée *et al.* (1995, 1996) for plant communities.

INTRODUCTION

Nutrient availability and nitrogen speciation have been shown to play a decisive role in determining performance of marsh orchid species, both in experimental circumstances and in fertilization trials in the field (cf. Dijk *et al.* 1997). In particular, higher N levels were shown to affect growth of *Dactylorhiza* seedlings[1] negatively in non-symbiotic culture (Dijk & Eck 1995a). A clear differentiation among these species was shown with respect to nitrogen and phosphate optima in these circumstances. *Dactylorhiza praetermissa* (Druce) Soó showed preference for high N concentrations in comparison to *D. incarnata* (L.) Soó, *D. maculata* (L.) Soó and *D. majalis* (Reichb. f.) Hunt & Summerh. For the calcicole *D. incarnata* a pronounced toxicity of ammonium ions was demonstrated in non-symbiotic culture, especially at low pH (Dijk & Eck 1995b). To a lesser degree ammonium toxicity was also observed in seedlings of the lesser calcicole *D. maculata* (Dijk *et al.* 1997). These basal responses in non-symbiotic culture can be modified, however, as a result of mycorrhizal infection, which tends to shift the optimum in nutrient response at lower concentrations. Growth of marsh orchid species was shown to be facilitated at relatively low N concentrations, while at higher concentrations some mycorrhizal strains may induce increased mortality (Dijk & Eck 1995c). At increased N concentrations (both as nitrate and ammonium) symbiotic development of seedlings of this species is disturbed (Beyrle *et al.* 1991).

Less is known about differentiation between orchid species with respect to phosphate requirements. *D. maculata* ssp. *maculata* and *D. praetermissa* were shown to grow best at relatively low phosphate levels in non-symbiotic culture (Dijk & Eck 1995a). Phosphate metabolism of orchids is highly affected by infection by symbiotic fungi. Mycorrhizal infection has been shown to facilitate growth of *D. praetermissa* and *Goodyera repens* at low phosphate availability, both in the heterotrophe youth phase (Smith 1966), as in adult plants (Alexander *et al.* 1984). However, 2-year-old symbiotically grown *D. majalis* juveniles grown in pot culture without P-shortage grew equally well whether or not the external mycelium was drenched in fungicide (Hadley & Pegg 1989). Supplying moderate amounts of N and P to adult plants of *D. fuchsii* in monoculture reduced growth, while *Orchis morio* did not respond (McKendrick 1996).

Negative effects of increased nutrient levels on orchid performance have also been demonstrated in the field. Although insensitive to moderate P fertilization in experimental circumstances (McKendrick 1996), fertilization of relatively productive hay meadows with high levels of mineral phosphate did decrease the number of flowering individuals of *Orchis morio* (Silvertown *et al.* 1994). This suggests that negative effects on performance of this species operates at least partly by shifting competition equilibria. N and P fertilization decreased performance of *D. majalis* in *Calthion palustris* hay meadows, in contrast to the application of K (Dijk & Olff 1994). Negative effects of fertilizer application on orchid performance were disproportional to vegetation changes,

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[1] Stages of development are defined as follows: *protocorm*: heterotrophic youth stage prior to the formation of the first shoot; *seedling*: plants with protocorm and the first shoot, but still lacking the first genuine tuber; and *juvenile*: plants having passed the seedling stage, with a first simple genuine tuber but before the first flowering. The term *adults* refers to plants having passed the juvenile stage, with bifurcate tubers and which are able to flower.

suggesting that, besides changes in competition equilibria, direct toxicity of high nutrient levels might also play a role.

Information on orchid nutrient responses, as summarized above, is therefore largely based on data obtained in highly arteficial circumstances, mainly in *in-vitro* culture. The present study was designed to check whether the nutrient availability and the plant responses to it in an experimental setting may explain distribution patterns of marsh orchid species in natural habitats. In a hay meadow in which populations of four *Dactylorhiza* species occurred, spontaneous distribution patterns were related to soil nutrient availability and to the corresponding vegetation composition.

In hydrologically undisturbed fens and fen meadows, relatively stable gradients in soil fertility are often associated with relatively stable gradients in groundwater flow patterns and pH (Wassen & Joosten 1996; Wassen *et al.* 1996), resulting from complex hydrological gradients with discharge of groundwater from various sources (Grootjans *et al.* 1988; Kemmers & Jansen 1988; Van Wirdum 1991; Everts & De Vries 1991; Wassen *et al.* 1992). Several marsh orchid species occupy characteristic positions in these hydrologically controlled gradients (Succow 1971). Therefore, distribution patterns were also related to the ionic composition of groundwater.

The marsh orchids studied here are slowly developing species that may need 4 years to reach the flowering stage (Vanhecke 1991). In particular, symbiotically growing individuals in the youth phase may be expected to tolerate a less broad range of nutrient concentrations than adult plants (see McKendrick 1996; Dijk *et al.* 1997). Since *Dactylorhiza* species are long-living (Tamm 1972, 1991), the actual distribution patterns in the field may therefore reflect the response of developing seedlings to the prevailing edaphic circumstances decades before the actual field observations. To minimize effects of possible changes in edaphic circumstances during population development, field responses expressed as differences in distribution patterns were checked by determining survival of juveniles in the field.

MATERIALS AND METHODS

Study area

The study was carried out in 'de Hazematen', a species-rich meadow reserve along the 'Lieversche Diep' (53°08′ N 6°35′ W) near Roden (Fig. 1; see also Grootjans *et al.* 1988). The valley is surrounded by forests and heaths on sandy soils. A thin boulder clay and a deeper, fine 'pottery clay' layer are present in the subsoil at the valley flanks, but they are not completely impervious to water flow. Mineral-poor subsurface water flows from the valley flanks. In the valley itself, both clay layers are dissected by a former stream bed; consequently calcareous groundwater from a deep aquifer (*c.* 50 m below the surface) can reach the soil surface here. The soil mainly consists of fen peat, up to 4 m thick. Small sand dunes occur in the valley, often covered by a thin layer of dessicated peat. Large parts of the meadows can be classified as *Angelico-Cirsietum oleracei*; a relatively basiphilous community of the *Calthion palustris* alliance (Schaminée *et al.* 1996). *Polygonum bistorta* and *Carex acutiformis* are frequent in this meadow type. Close to the valley flanks poor fen (*Caricion nigrae*) and relics of mesotrophic fen meadow (*Junco-Molinion*) still exist. Locally reeds and alder carrs are present.

Four *Dactylorhiza* species occur in the Hazematen meadows: *D. maculata* (L.) Soó ssp. *maculata*, *D. praetermissa* (Druce) Soó, *D. majalis* (Rchb.) Hunt & Summerh. and *D. incarnata* (L.) Soó.

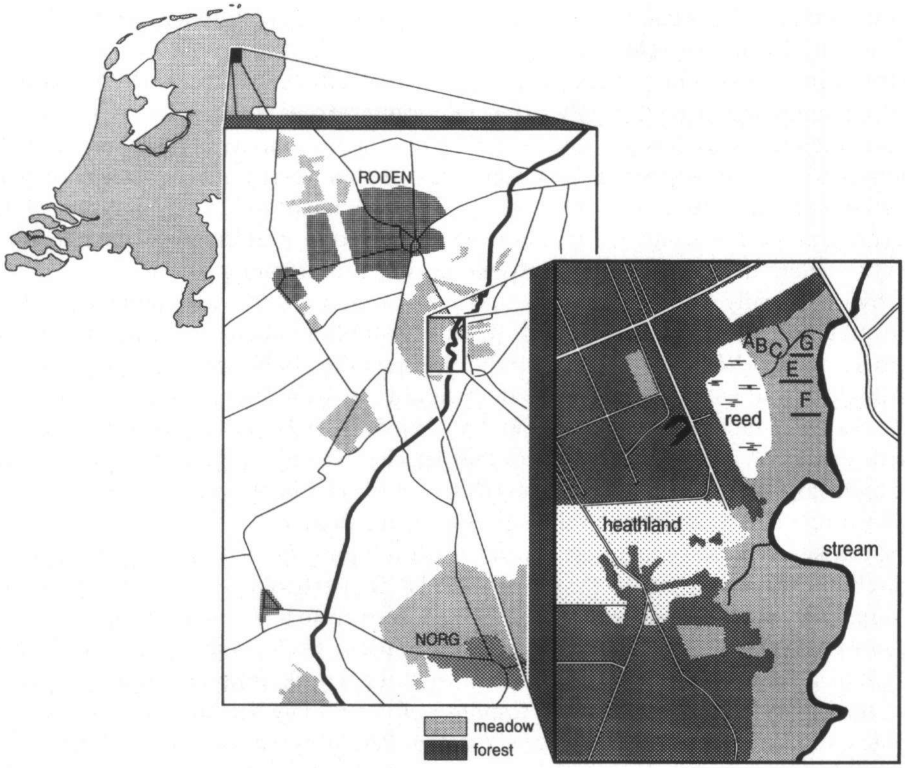

Fig. 1. Location of the study area 'de Hazematen', and location of the experimental plots.

Vegetation composition

To characterize the phytosociological position of the various orchid species 44 vegetation releveés were made in June and July (1991–94) using a modified Braun–Blanquet cover–abundance scale. The plots were sized 1 × 1 m. Moss species were disregarded.

Differences between orchid taxa in phytosociological context were analysed by subjecting square-root transformed coverages to a detrended correspondence analysis (DCA; Ter Braak, 1987). Detrending of ordination scores took place by division in 26 segments. Species occurring only once in the dataset were removed from the analysis.

Four plots with the highest abundances of one of the orchid species were chosen as experimental sites for introducing seedlings and measuring soil and groundwater conditions. Two contrasting sites in which no orchids occurred were also chosen as experimental sites. The position of the experimental plots is shown in Fig. 1. The vegetation zone containing plot B formed the home site of *D. maculata* ssp. *maculata*; in this site scattered individuals of all other marsh orchid species also occurred. The zone around plot G had the highest numbers of *D. majalis*, while other species were nearly absent. Plots C and F formed the home site of *D. incarnata. D. praetermissa* occurred sporadically in the margins of the fen peat; its home site lay some distance from the experimental plots.

Groundwater composition

In each experimental plot three nested piezometres were placed, with the aim to illustrate the relatively large-scale differences in groundwater feeding underlying the meadow system as a whole. The PVC tubes (Ø 40 mm) had filters at 30, 80 and 160 cm below the soil surface. Groundwater samples were taken in June, August and November 1991 and February and May 1992. Tubes were emptied 1 or 2 days before sampling to allow refilling with fresh groundwater. Samples were divided in two fractions, of which one (50 ml) was used to determine Ca^{2+}, Mg^{2+}, Na^+, K^+ and Fe content; these were acidified to pH 2 by adding 2·5 ml 4% HCl. Cation contents were determined by means of an Atom Absorbance Spectro-photometer (AAS). In the remaining subsample (100 ml) concentrations of NO_3^-, NH_4^+, Cl^-, SO_4^{2-} and HCO_3^- were measured. NO_3^- and NH_4^+ were determined by measuring the diazo-complex at 540 nm and Na-salicylate at 660 nm using a continuous San+ segmented flow autoanalyser (Skalar). Concentrations of Cl^- and SO_4^{2-} were determined using an autoanalyser by measuring ferric thiocyanate colour at 490 nm, and methylthymol blue colour at 460 nm, respectively. Concentration of HCO_3^- was determined by titration with 1 M HCl up to pH 4·5. Quantities of NO_3^-, NH_4^+, HCO_3^-, EC_{25} and pH were determined within 24 h after sampling. To check for the reliability of the analyses, the charge balance and EC_{25} were calculated. Unreliable analyses – a deviation of the charge balance of more than 10% from electro neutrality – were discarded.

The main dimensions of variation in the groundwater samples were summarized by subjecting the data to principle components analysis (PCA) in which a Varimax rotation was applied to maximize differences of scores on the axes.

Chemical analysis of soil samples

In June 1991, soil samples of the top layer (15 cm) were taken in the immediate vicinity of the various experimental plots. Coarse root fragments and living material were removed prior to further analysis. Organic matter content was determined by weight loss on ignition at 500°C for 3–4 h. Quantities of exchangable Ca^{2+} and K^+ were determined using an AAS after overnight extraction in 0·5 M NaCl for Ca^{2+} and for 1 h in HCl-oxalic acid for K^+. Soil total N content was determined by the Kjeldahl–Lauro method. Total P was determined by digestion with H_2SO_4, HNO_3 and $HClO_4$, and subsequent colorimetric analysis of PO_4^{3-} using NH_4MoO_4. Contents of available phosphate (P_{AL}) were assessed by means of extraction for 4 h in 0·1 M ammonium lactate and 0·4 M acetic acid. NO_3^- and NH_4^+ were determined as above using an autoanalyser after overnight extraction in 1 M KCl in fresh soil within 24 h. Soil pH (pH_{KCl}) was determined in fresh soil after standing overnight.

Correlations between measured soil parameters, and plot differences therein, were summarized using a PCA as above.

Soil fertility and nutrient limitation

Fertility and the extent of nutrient limitation of the same soil samples were determined by means of a bioassay using *Cirsium palustre* (L.) Scop. as a phytometer. This rapidly growing species occurs frequently over the whole gradient and could therefore be expected to grow on all soil samples. Moreover, when grown on the original soil turfs, it was shown to respond to extra N-, P- and K supplied by nutrient solutions (Pegtel,

Table 1. Composition of basal medium (a) for non-symbiotic *in-vitro* culture of *Dactylorhiza* spp., and (b) added macronutrients and medium pH before autoclaving for resp. *Dactylorhiza maculata* (1), *D. majalis* and *D. praetermissa* (2) and *D. incarnata* (3)

	$(g\,l^{-1})$		(mM)		(μM)
(a) Basal medium					
Agar	6·0	KH_2PO_4	0·4	Na_2EDTA	140
Sucrose	10·0	$MgSO_4$	0·5	$FeSO_4$	100
Malt agar	2·0			$MnCl_2$	33
Charcoal	0·2			$ZnSO_4$	2·8
				$NaMoO_4$	1·0

(b) Other macronutrients (in mM) and medium pH	(1)	(2)	(3)
K_2SO_4	1·0		
$CaCl_2$	1·0	1·0	
$Ca(NO_3)_2$			1·0
KNO_3	4·0	6·0	4·0
NH_4NO_3	0·5	6·0	2·0
$(NH_4)_2SO_4$	2·0		
Medium pH	5·5	6·0	7·0

unpublished data). Hence, the species satisfies the most important requirements for a suitable phytometer (Pegtel 1987).

Plastic pots 9 cm in diameter were filled with equal volumes of soil of the various experimental sites. Seeds of *C. palustre* were germinated until appearance of the second leaf, on Petri dishes subjected 16 h to light at 25°C and 8 h of darkness at 15°C. At that moment a single seedling was transferred to each pot. Samples of the various sites were divided into four groups, to which different nutrient solutions were added twice a week in order to determine the extent of N-deficiency relative to that other nutrients (P, K, Mg and Ca). Two factors were varied simultaneously in the nutrient solutions in a three-way factorial design: addition or exclusion of 5 mM nitrogen (as NH_4NO_3), and incorporating or omitting a mixture of other macronutrients (1·5 mM KH_2PO_4, 1·75 mM K_2SO_4, 0·75 mM $MgSO_4$ and 3 mM $CaSO_4$). Each experimental group was replicated six times. Plants were grown in a greenhouse in late spring and early summer 1991; day temperatures did not fall below 17°C during the day and 10°C at night, but otherwise followed the natural fluctuations.

After 3 months of culture, aboveground biomass was harvested and dried overnight at 70°C. Dry weights were analysed statistically by applying an analysis of variance (ANOVA) on log-transformed yields, the latter to meet demands for a normally distributed test variable and homogeneity of variance.

Orchid seedling survival in experimental plots

Sterilized seeds of *Dactylorhiza incarnata*, *D. maculata* ssp. *maculata*, *D. majalis* s.s. and *D. praetermissa* were germinated non-symbiotically in the dark. Germination procedure and media composition have been given elsewhere (Dijk & Eck 1995a,b). After germination in the dark, seedlings were replated in polypropylene vessels and subjected to a diurnal rhythm of 16 h light at 22°C and 8 h of darkness at 18°C. *D. majalis* and *D. praetermissa* seedlings were grown on the standard medium (Table 1, (2)). Media for *D. maculata* and *D. incarnata* were modified with respect to nitrogen sources and pH (Table 1, (1) and (3)). Seedlings were replated on fresh media every 3 months for a period of at least 1·5 years, until the first genuine tuber had developed. Subsequently cultures were chilled for 3 months at a constant temperature of 5°C to break shoot dormancy, under a diurnal rhythm of 12 h of light and 12 h of darkness. Afterwards, the cultures were put back under the same conditions as before the chilling period. When growth resumed, the agar medium in the vessels was removed and replaced by a mixture of sand and black peat in the ratio 1:1 to which, with the exception of *D. maculata*, some lime was added. When growth continued, plants were slowly hardened by opening the vessel covers little by little. Subsequently, plants were repotted on the same soil mixture and placed outdoors. In the winter plants were kept in the greenhouse at a minimum temperature of 5°C. Most individuals of *D. majalis* and *D. praetermissa* were 4 years old, and those of *D. incarnata* and *D. maculata* 3 years old when transferred to the field.

The orchids were placed in the experimental plots in the field in June 1991. Holes 6 cm in diameter were drilled within each experimental plot in the top 10 cm of the soil. Plants of known fresh weight were clamped in the split soil core still bearing the original vegetation, and put in half-open, slotted pots allowing entrance of phreatic water and access of roots of natural competitors. The pots were put back into the original drill holes, where they remained for 1 year. Ten individuals of *D. majalis* and *D. praetermissa* were introduced per experimental plot. Due to shortage of material, less individuals of *D. incarnata* and *D. maculata* could be tested, with a minimum of five per plot. In June 1992 plants were harvested, and survival and changes in fresh weights determined.

RESULTS

Vegetation composition of the experimental plots

Plots A, B and C, situated in the lower parts of the gradient at the valley flank, could all be classified to the *Caricion nigrae*. Differences existed, however, between the plots (Table 2); plot A had a higher share of eutraphent grasses (*Agrostis canina* and *Calamagrostis canescens*), while plot B had the highest abundance of less eutraphent *Junco-Molinion* species (*Carex panicea*, *Dactylorhiza maculata* and *Succisa pratensis*). Plot C was characterized by the occurrence of some basiphilous species (*Carex diandra*, *C. appropinquata* and *D. incarnata*). Plot E was situated on top of a small sandy dune and therefore drier. Its vegetation can be considered a *Festuca rubra–Lotus uliginosus Calthion* frame community. Plots G and, especially, F were situated in moister sites with tall herbs and sedges, and belonged to the *Angelico-Cirsietum oleracei*, a highly productive, relatively basiphilous meadow community.

Phytosociological position of the orchid species

The main dimension of vegetation difference within the dataset (Fig. 2) mirrors the occurrence of the plant species from the valley flank (low scores) towards the centre (high scores). Low axis 1 scores are assigned to species characteristic of low productivity fens (*Caricion nigrae*) and fen meadows (*Junco-Molinion*). Species characteristic of more productive grasslands (*Arrhenatherion elatioris*) are attributed high axis 1 scores. On the second axis different types of waterlogged communities are separated. Low scores

Table 2. Occurrence of the most discriminating plant species in the experimental plots on a gradient in the Hazematen hay meadows. Vegetation composition was recorded in duplo at both sides of the experimental plots. Abundances are given in the decimal scale and an estimate of the coverage in percentages

	A1	A2	B1	B2	C1	C2	F1	F2	G1	G2	E1	E2
Caricion nigrae												
Carex echinata	1	—	2	2	—	—	—	—	—	—	—	—
Agrostis canina	1	4	1	1	—	—	—	—	—	—	—	—
Hydrocotyle vulgaris	1	1	4	—	—	—	—	—	—	—	—	—
Viola palustris	—	1	1	1	1	—	—	—	—	—	—	—
Junco-Molinion												
Juncus conglomeratus	2	1	1	—	—	—	—	—	—	—	—	—
Carex panicea	—	—	4	10	—	—	—	—	—	—	—	—
Dactylorhiza maculata	—	—	1	1	—	—	—	—	—	—	—	—
Succisa pratensis	—	—	—	10	—	—	—	—	—	—	—	—
Juncus acutiflorus	4	1	20	20	10	10	—	—	—	1	—	1
Valeriana dioica	—	—	1	4	4	2	—	1	—	—	—	—
Calthion palustris												
Dactylorhiza majalis	—	—	—	—	—	—	—	1	2	—	—	—
Lychnis flos-cuculi	1	1	1	—	2	1	—	1	1	1	—	—
Caltha palustris	1	—	—	—	1	1	1	1	2	10	—	—
Lotus uliginosus	1	—	2	—	12	20	—	1	—	1	—	—
Polygonum bistorta	—	—	—	—	1	—	40	40	2	1	—	—
Filipendula ulmaria	—	—	4	2	20	20	30	10	10	10	1	—
Rhinanthus angustifolius	—	—	—	—	—	—	—	1	10	7	—	—
Phragmitetea												
Carex acutiformis	30	20	10	4	—	—	—	—	—	—	—	—
Calamagrostis canescens	10	40	1	—	1	1	—	1	—	—	—	—
Phragmites australis	—	—	—	—	7	4	—	—	2	4	—	—
Equisetum fluviatile	1	—	1	2	1	1	4	10	1	1	1	1
Molinio-Arrhenatheretea												
Festuca rubra	—	12	2	2	2	1	2	10	1	20	30	—
Holcus lanatus	1	1	1	—	—	1	10	20	12	20	30	30

on this axis are assigned to eutrophic tall sedge communities (*Magnocaricion*). Species of low productivity, small sedge communities (*Caricion nigrae*) as *Menyanthes trifoliata*, *Carex diandra* and *C lasiocarpa* are given high axis 2 scores. In the study area the four *Dactylorhiza* species occur in distinct variants of *Calthion palustris* meadows and transitions to small sedge marshes of the *Caricion nigrae. D. praetermissa* and especially *D. maculata* are assigned the lowest axis 1 scores. *D. maculata* has its optimum in transitions between *Crepido-Juncetum acutiflori* and *Carici curtae-Agrostietum caninae* communities, while *D. praetermissa* is most typical for *Calthion palustris* stands in which many tall herbs and sedges such as *Filipendula ulmaria* and *Carex acutiformis* occur. *D. majalis* was assigned a higher axis 1 score: although it occurred in several types of the *Calthion palustris*, it showed an optimum in the drier stands. *D. incarnata* was separated clearly by its higher axis 2 scores; it occurred most frequently in transitions between basiphilous low sedges and *Angelico-Cirsietum oleracei* stands.

Groundwater composition

In the PCA of parameters measured in the groundwater samples (Fig. 3), the two main extracted axes together explained 65% of the observed variation in the parameters.

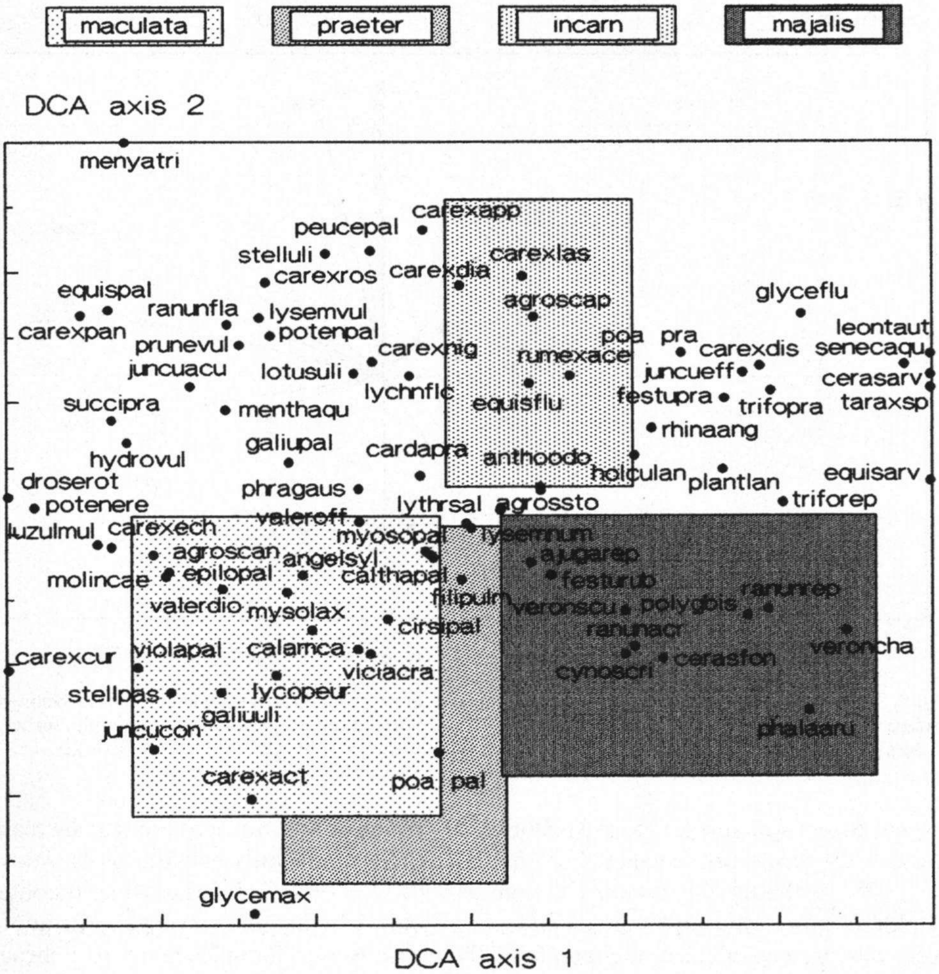

Fig. 2. Species scores on the first two axes extracted by detrended correspondence analysis (DCA) of 44 vegetation samples at the hay meadow complex 'de Hazematen'. Rectangles indicate ranges delimited by mean score ± tolerance for respectively *Dactylorhiza incarnata*, *D. maculata*, *D. majalis* and *D. praetermissa*; they give a summary of the most characteristic accompanying plant species of the four marsh orchid species. Names of the plant species have been abbreviated to the first five characters of the generic names and the first three of the species name; note 'carexact': *Carex acutiformis*; 'carexdit': *Carex disticha*; 'carexpan': *Carex panicea* and 'stellpas': *Stellaria palustris*. Eigenvalues of the axes: first axis 0·40; second axis 0·15.

The first axis showed high correlations with Ca^{2+}, HCO_3^- and Mg^{2+} concentration. Furthermore, a weaker correlation with pH was observed. The main dimension of variation in groundwater composition is therefore formed by the share of base-rich groundwater in the samples. The second axis showed high loadings for the major nutrients, i.e. phosphate, potassium and ammonium. Moreover, this axis was correlated strongly to the iron content of the groundwater samples. Na^+ and Cl^- concentrations were correlated strongly to the first axis, but also significantly to the second. Therefore, Na^+ and Cl^- levels reflect both discharge of base-rich groundwater and nutrient load.

A clear trophic gradient (E, A, B, F, C–G) was shown in the deep samples drawn at

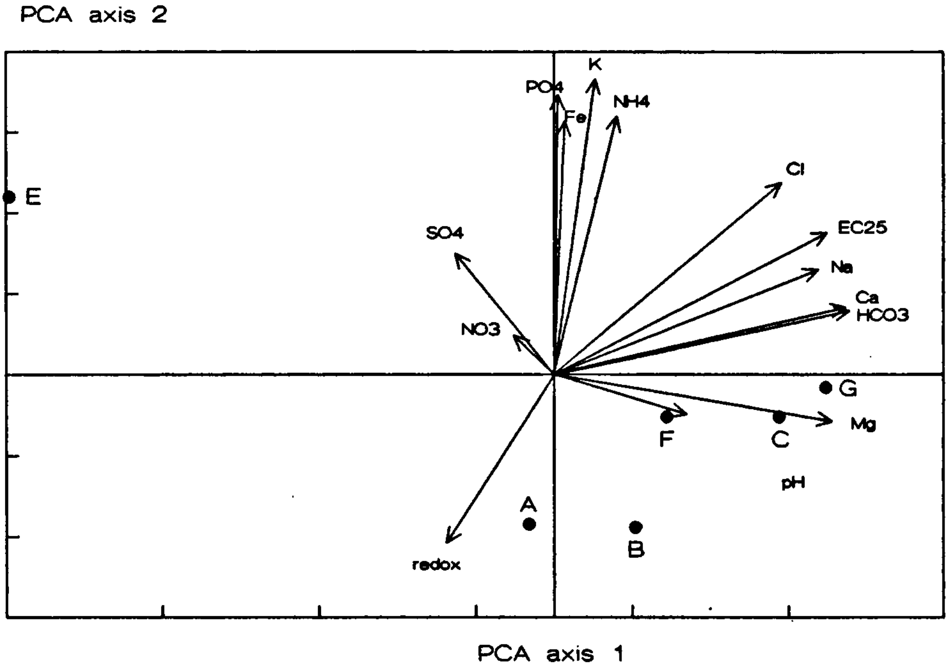

Fig. 3. Main dimensions of variation in ionic composition of groundwater samples at 160 cm below soil surface, as given by principal components analysis (PCA). Correlations are based on those samples for which a complete ionic analysis was available only. Eigenvalues of the axes: first axis 6·3; second axis 2·8.

160 cm below soil surface (Fig. 3). Both Ca^{2+} contents and nutrient content increased towards the centre of the valley. Ca^{2+} levels differed significantly between plots (ANOVA; F=23·35, P<0·001) and between sample periods (F=7·88, P<0·001). Mineral content in plot E was lower, and that in plots F, C and G higher than average. An annual cycle was present in calcium contents of the groundwater. Samples taken in February and May showed significantly lower, and those of June and August significantly higher calcium levels than average (data not shown). Increase of calcium levels in summer was stronger in plot E, and less pronounced in plot F. Decrease in winter was disproportionally strong in plot A but less in plot C.

Chemical analysis of soil samples

The first two PCA axes accounted for 82% of the total variation in the soil parameters (Fig. 4). The first axis (EV 6·7) accounted for a much larger fraction of the variation than the second (EV: 1·5).

At the time of sampling, mineral nitrogen in the soil samples was almost entirely present as ammonium ions. Total N, NH_4^+ and exchangable K^+ content of the soil samples were correlated strongly to the amount of organic matter. These factors were assigned high loadings on the first PCA-axis that summarizes the main dimension of difference in soil parameters. Plot C was assigned the highest score on this axis; the soil at this site is rich in organic matter and therefore exhibits high nitrogen and potassium contents. Plots B, E and G (low scores on axis 1) were relatively poor in organic matter, ammonium and potassium. Plots A and F had intermediate scores.

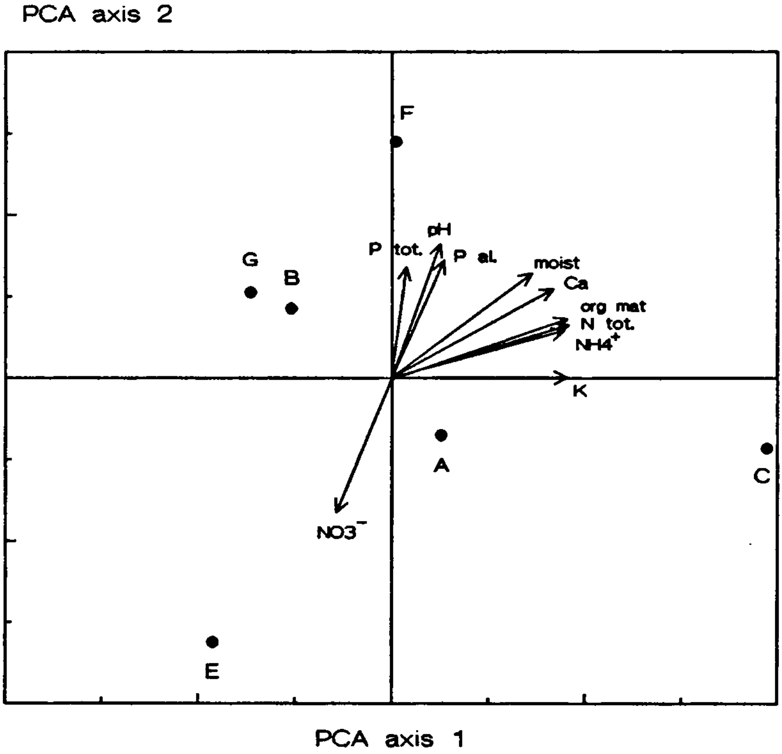

Fig. 4. Main dimensions of variation in measured soil parameters at the six experimental plots as given by principal components analysis (PCA). Eigenvalues of the axes: first axis 6·7; second axis 1·5.

The second axis showed high positive loadings for total phosphate and available phosphate (P_{AL}), and a strongly negative one for nitrate concentration. As expected, nitrate level was negatively correlated with soil pH and moisture content. Available and total phosphate concentration were positively correlated with soil pH. This correlation originates partly from the extremely low phosphate content, and low pH and calcium content of the soil observed in plot E as a result of low water tables. Nitrate levels in plot E were absolutely low, but relatively much higher than those of the other plots. Plot F had the highest score on the second axis, its soil samples showing high quantities of both total and available phosphate, and an intermediate soil pH. The remaining plots (A, B, C and G) were assigned intermediate scores on the second axis. Plot G showed high total P contents, but it differed from all other plots with exception of E by its very low concentration of available P.

Soil fertility and nutrient limitation

Pronounced differences in yield were observed between *Cirsium palustre* plants grown on soil samples of the various plots in the control treatment; these indicate differences

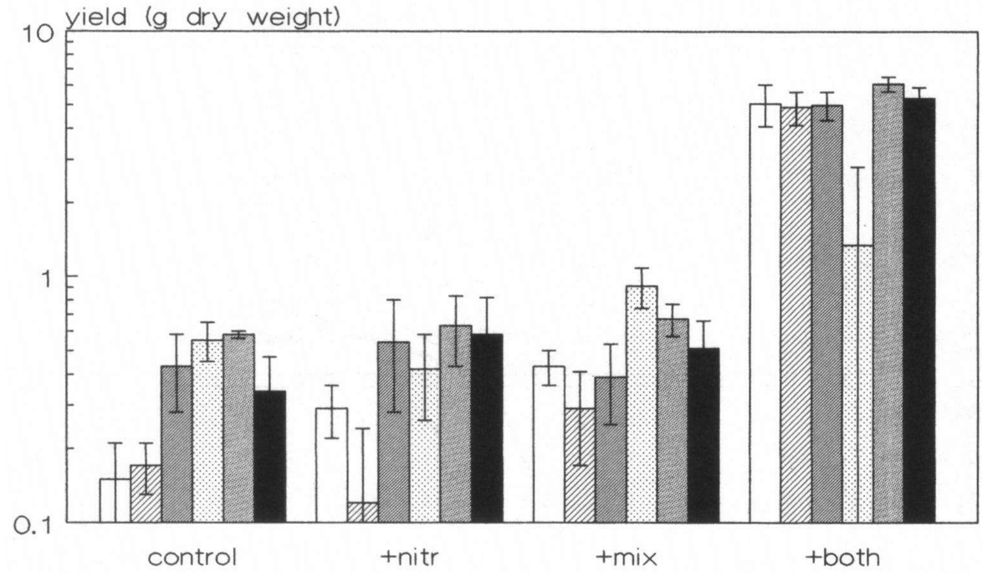

Fig. 5. Dry weights of *Cirsium palustre* grown on soil samples from six experimental plots as dependent on nutrient additions in the bioassay of soil fertility and nutrient limitation. Error bars indicate 95% confidence intervals of the estimates. Note the log-scale on the *Y*-axis. Nutrient treatments are coded as follows: C: unfertilized control; N: nitrogen; MIX: mixture of the other major nutrients (P, K, Ca, Mg) and N+MIX: complete fertilization of both nitrogen and other macronutrients. Plots: ☐, A; ▨, B; ▩, C; ■, E; ■, F; ■, G.

in overall soil fertility along the gradient (Fig. 5). Plots A and B were significantly less fertile, and plots E and F were more fertile than average.

Dry weights of plants were affected significantly by the nitrogen level in the nutrient solution (Table 3), demonstrating the suitability of the test species as a phytometer for nitrogen availability of the soil. In general the soil samples were nitrogen limited to some extent, plots A, C and G more pronounced, while in plot E N limitation could not be demonstrated. Yields were, however, affected even stronger by the presence or absence of the mix of other macronutrients in the applied nutrient solution. Soil samples are therefore limited to a larger degree by at least one of the macronutrients present in the mixture, and the test species responded to availability of this nutrient. The plots at the valley flank (A and B) were significantly more limited by this factor, while plants grown on soil of plot E were hardly inhibited by low levels of this nutrient. The pattern in extent of limitation by this factor, matched the total P content of the soil samples. In particular in plot B, growth limitation by this nutrient was strong in comparison to N limitation, the latter being expressed only if the other limitations were cancelled.

Overall yields of *C. palustre* over the dataset as a whole were lowest in plot B and E (Table 3, main effect); for plot B probably as a result of the multiple nutrient limitations. For plot E low overall yield originates from lack of response to the nutrient additions, be they nitrogen, the mix of other nutrients or the complete solution, implying that biomass must be limited by other factors than nutrient shortage, presumably its marked low pH.

Table 3. ANOVA table for bioassay of soil fertility and nutrient limitation of soil samples across an environmental gradient at 'de Hazematen', using log-transformed dry weights of the phytometer *Cirsium palustre* as the dependent variable. Overall significances of factors are given in section (a), in which 'NITR' encodes the effect of NH_4NO_3 addition, 'MIX' that of addition of a mixed solution containing P, K, Ca and Mg. Plot differences are given in section (b); the main effect 'PLOT' gives overall differences in yield of *C. palustre*, averaged over all nutrient treatments; the interaction terms 'PLOT*NITR', 'PLOT*MIX' and the third order interaction term indicate the extent of limitation by nitrogen, other main macronutrients and the interaction between both, respectively. The more positive the estimate, the higher the gain in biomass of the phytometer on soil of a given plot as a result of the nutrient application, and hence the stronger the limitation of the particular plot for the nutrient. Factors and interactions not comprising 'PLOT' do not apply in this section. Significances are expressed as *t*-values of the deviations from the mean response of the phytometer. 'df' denotes degrees of freedom. Significances are coded as follows: ***: $P<0{\cdot}001$; **: $0{\cdot}001 \leqslant P<0{\cdot}01$; *: $0{\cdot}01 \leqslant P<0{\cdot}05$

Factor name	df	*F*	Sign.
(a) Significances of factors			
NITR	1	436·4	***
MIX	1	545·1	***
NITR*MIX	1	383·1	***
PLOT	5	11·0	***
PLOT*NITR	5	18·9	***
PLOT*MIX	5	9·4	***
PLOT*NITR*MIX	5	12·7	***

Plot	A	B	C	E	F	G
(b) Yield differences between plots						
PLOTS	−1·19	−3·06**	1·38	−4·41***	5·41***	2·07*
PLOT*NITR	2·13*	1·63	2·02*	−9·70***	1·59	2·42*
PLOT*MIX	2·63*	2·55*	−0·47	−6·43***	0·96	0·75
PLOT*NITR*MIX	1·00	2·82**	1·61	−7·83***	0·83	0·69

Survival of orchid seedlings

As a result of high mortality, orchid yield was distributed strongly bimodally, resulting in biased yield analyses. Therefore, only seedling survival was analysed by means of a log-linear test (Christensen 1990), in which site and orchid species were used as explanatory variables and survival as the target variable (Table 4). Survival was significantly, but to only a minor extent, determined by the fresh weight of seedlings at the start of the experiment: the higher the starting weight, the higher the chance of surviving (logistic regression: $R=0{\cdot}14$; $0{\cdot}05<P\leqslant0{\cdot}01$). Flowering incidence of surviving individuals in the following year was correlated more strongly by fresh weights of the individuals at the start of the experiment ($R=0{\cdot}42$; $P<0{\cdot}001$). One year after transfer to the field, significant differences in seedling survival were observed between species and between plots (Fig. 6). Mean survival of *D. majalis* and *D. praetermissa* seedlings was higher than that of *D. incarnata* and *D. maculata*. Since the difference can be attributed only to a limited extent to differences in starting weight, this means that *D. incarnata* and *D. maculata* are more critical than the other two marsh orchid species.

The general response was one of significantly higher survival in plot F, characterized by high water tables, base-rich groundwater and a high soil fertility. In plot E, characterized by the lowest Ca^{2+} contents in the groundwater and high soil fertility,

Table 4. Log-linear analysis of seedling survival of four *Dactylorhiza* species after 1 year's stay on various plots on a gradient in de Hazematen. 'SURV' gives significance of the frequency differences between survival classes 'dead' and 'survivor'. Interactions encompassing the target factor 'SURV' give significance of survival differences attributable to the explanatory factor (e.g. 'SPECIES*SURV': survival differences between species); the remaining explanatory ones those of frequency differences within classes determined by the experimental setup (e.g. SPECIES*PLOT differences in number of explanted individuals per plot). For coding of significances and abbreviations see caption above Table 3

Factor name	df	χ^2	Sign.
SURV	1	0·83	NS
SPECIES	3	11·72	**
PLOT	5	2·82	NS
SPECIES*SURV	3	47·24	***
PLOT*SURV	5	14·98	*
SPECIES*PLOT	15	19·70	NS
SPECIES*PLOT*SURV	15	43·11	**

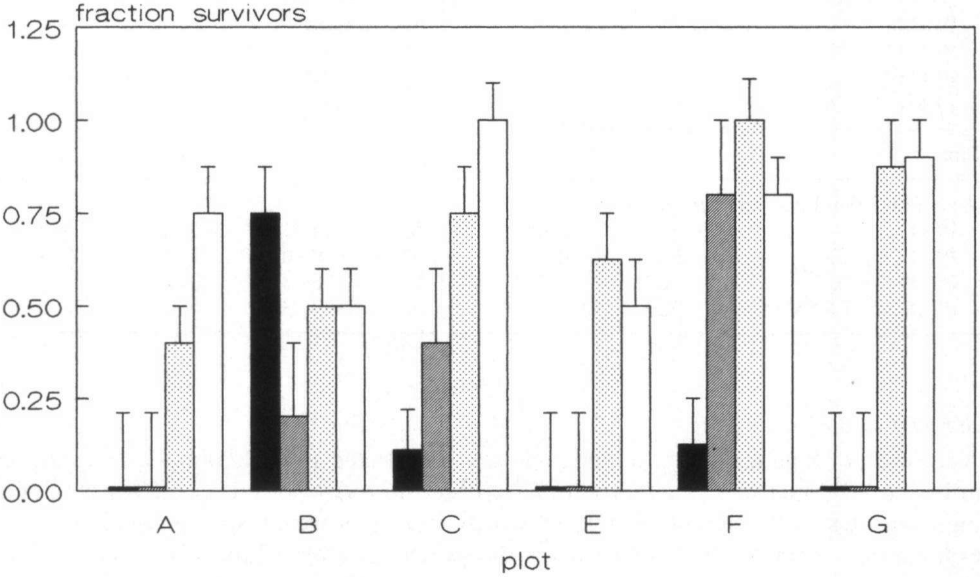

Fig. 6. Survival of the four *Dactylorhiza* species across the experimental gradient. Survival is expressed as the fraction of total number; bars indicate standard errors of the fraction. ■, *Dactylorhiza maculata*; ▨, *D. incarnata*; ■, *D. majalis*; □, *D. praetermissa*.

survival of orchid seedlings was generally lowest; only small and unhealthy-looking seedlings of *D. majalis* and *D. praetermissa* remained after 1 year.

Interspecific differences in survival within the various plots occurred, however; where differences were significant, seedlings survived better in the plots closest to their home sites. Relatively high survival of *D. maculata* was observed in plot B, at mediocre Ca^{2+} levels in the groundwater and at low soil fertility. In the same plot, survival of *D. praetermissa* was relatively low. In plot A, under the same hydrological conditions and similar P, but higher N contents of the soil, the response of *D. maculata* was equally poor, and that of *D. praetermissa* equally well, as in the remaining plots. Survival of *D. majalis* and *D. incarnata* was not different from the general response: like all tested species survival was high in plot F and low in plot E.

DISCUSSION

The main vegetation differentiation in the study area is associated with difference in groundwater feeding, which is largely reflected by differences in calcium content of the groundwater at 160 cm below the surface. The groundwater near the valley flanks has relatively low calcium levels, indicating a predominant influence of base-poor subsurface water from a local infiltration area over base-rich groundwater from a deep aquifer. This interpretation is also in agreement with more detailed results given previously (Grootjans *et al.* 1988). Almost the entire transect is influenced by base-rich groundwater during the summer, with the exception of a drained site in which base-rich water does not reach the upper soil layer. High SO_4^{2-} and NO_3^- contents suggest oxidation and mineralization of organic matter here due to low water levels. Other parts in the centre of the valley experience the highest seepage intensity and are influenced by base-rich groundwater, both in winter and in summer. Fertility and soil nutrient content were found to be related to these hydrological conditions. Total N and exchangable NH^+ and K^+ in the soil were correlated to the amount of organic matter, which was high in the wetter, peaty sites and low in the drier plots. Mineral nitrogen was largely present as NH_4^+, due to prevailing wet, and hence anaerobic conditions. Apart from that, nitrate concentrations can be expected to be low as a result of preferential NO_3^- uptake by the vegetation during the sampling time, in the active growing season. High total soil P contents were found in areas influenced by seepage of calcareous and iron-rich groundwater, probably by continuous precipitation of iron phosphate complexes. Availabily of P is lower in the drier plots, probably by phosphate immobilization by Fe^{3+} (Patrick & Khalid 1974).

The discharge of base-rich seepage water plays an important role in the spatial distribution and survival of marsh orchids in the field, in interaction with soil ammonium and phosphate levels. Patterns in natural distribution and in seedling survival with respect to these factors can, for the most part, be explained by the nutrient responses of the species obtained in experimental conditions. Survival of all orchid species is low in drained sites with dessicated peat layers. Soil fertility appears not to be limited by the main nutrients here. Consequently another factor must limit plant growth, presumably a low pH or iron deficiency (compare Janiesch 1980). The corresponding vegetation in these conditions can be considered a degraded *Calthion palustris*, in which all characteristic species disappeared. None of the orchid species occurred spontaneously in these frame communities.

The occurrence of *D. maculata* and *D. praetermissa* shows a relationship with a low total P content of the soil influenced by groundwater with a moderately high Ca^{2+} content. The low P optima of these species are confirmed by earlier results in nonsymbiotic *in-vitro* culture (Dijk & Eck 1995a). Survival of *D. praetermissa* seedlings demonstrates a preference for the higher N (NH_4^+) levels in these hydrological conditions in comparison to soils under the same conditions, but with lower N contents. Exactly the opposite pattern was observed in *D. maculata*, which showed high survival only when N contents were relatively low. The suggestion that the difference in field response is attributable to differences in soil N-availability is confirmed by experimental studies in axenic *in-vitro* culture, showing a low optimum with regard to N-availability for *D. maculata*, and a strikingly high one for *D. praetermissa* (Dijk & Eck 1995a). The tolerance of *D. praetermissa* for high N-availability might well be responsible for its success in the most eutrophic *Calthion* communities on peat meadows (Schaminée *et al.* 1996) and disturbed sites. *D. maculata* optimally occurs in vegetation in which species of the *Caricion nigrae* and *Junco-Molinion* occur. The overall soil fertility, as indicated by the bioassay, is low and is limited primarily by a major nutrient other than nitrogen, probably phosphate. The low N optimum of *D. maculata* seems largely determined by its response to ammonium ions, since higher N levels are tolerated when the ratio between NO_3^- and NH_4^+ is high (Dijk *et al.* 1997). The preference of this species for high NO_3^-/NH_4^+ ratios in soil water, low soil P concentrations and a rather high soil pH has also been established by Roelofs *et al.* (1996).

The remaining two marsh orchids are restricted to areas with seepage of the most calcareous groundwater. *D. majalis* occurred predominantly in the drier *Calthion palustris* stands, associated with a high seepage intensity, high soil fertility limited by nitrogen and a large phosphorus pool. The available fraction of the phosphorus pool at the home site of this species was small, however, due to immobilization of phosphate as a result of high iron contents of the soil. In *Calthion palustris* hay meadows, the species appeared to be inhibited by both N and P fertilization in field fertilization trials (Dijk & Olff 1994). In asymbiotic culture, however, the species showed optimal growth at intermediate N, but high P concentrations (Dijk & Eck 1995a). High phosphate requirements of the species could be met by mycorrhizal activity, or alternatively by means of the higher water tables during the winter period, releasing immobilized Fe(III)phosphate by reduction to its better soluable form (Fe(II)phosphate).

The distribution pattern of *D. incarnata* seems to be determined entirely by ammonium concentration in interaction with soil pH. In axenic culture, growth of this species was impeded by ammonium and by pH values below 6, while ammonium toxicity decreased with higher pH (Dijk & Eck 1995b). This phenomenon might account for the preference of the species in the field for areas with a high seepage intensity of base-rich groundwater. The species occurs mainly in transitions between basiphilous *C. lasiocarpae* stands and *C. palustris* communities. These waterlogged sites experience a pronounced influence of base-rich groundwater by exfiltration of groundwater or by infiltration of base-rich surface water. The soils have high contents of organic matter, and are therefore rich in NH_4^+ ions. Tolerance for ammonium will be increased in areas with base-rich groundwater, due to higher soil pH. In the less fertile fen meadow part at the valley flank, the species grows in low numbers in an area with exfiltration of base-poor groundwater and a lower soil pH. At lower NH_4^+ concentrations, resulting from the smaller soil nitrogen pool, *D. incarnata* appears to be able to tolerate less base-rich conditions as well.

The results of our field trials largely reflect the non-symbiotic nutrient responses obtained in experimental conditions. These have been shown to form the basis for nutrient responses of the mycorrhizal entity when grown symbiotically (Dijk & Eck 1995c). For *D. majalis* the field response, especially with respect to P-concentration, cannot be explained exclusively by axenic nutrient responses. In particular for this species, which has been shown to accept a wide range of contrasting endophytes (Harvais & Hadley 1967; Hadley 1970; Dijk & Eck 1995c), it would be rewarding to discriminate between distribution patterns of the separate, differently responding mycorrhizal associations of the same orchid species. In less selective orchid species, a broader overall tolerance might be realized by dissimilar nutrient optima of ecologically contrasting orchid–fungus associations.

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