Variation in flooding-induced morphological traits in natural populations of white clover (*Trifolium repens*) and their effects on plant performance during soil flooding

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- **Background and Aims** Soil flooding leads to low soil oxygen concentrations and thereby negatively affects plant growth. Differences in flooding tolerance have been explained by the variation among species in the extent to which traits related to acclimation were expressed. However, our knowledge of variation within natural species (i.e. among individual genotypes) in traits related to flooding tolerance is very limited. Such data could tell us on which traits selection might have taken place, and will take place in future. The aim of the present study was to show that variation in flooding-tolerance-related traits is present among genotypes of the same species, and that both the constitutive variation and the plastic variation in flooding-induced changes in trait expression affect the performance of genotypes during soil flooding.

- **Methods** Clones of *Trifolium repens* originating from a river foreland were subjected to either drained, control conditions or to soil flooding. Constitutive expression of morphological traits was recorded on control plants, and flooding-induced changes in expression were compared with these constitutive expression levels. Moreover, the effect of both constitutive and flooding-induced trait expression on plant performance was determined.

- **Key Results** Constitutive and plastic variation of several morphological traits significantly affected plant performance. Even relatively small increases in root porosity and petiole length contributed to better performance during soil flooding. High specific leaf area, by contrast, was negatively correlated with performance during flooding.

- **Conclusions** The data show that different genotypes responded differently to soil flooding, which could be linked to variation in morphological trait expression. As flooded and drained conditions exerted different selection pressures on trait expression, the optimal value for constitutive and plastic traits will depend on the frequency and duration of flooding. These data will help us understanding the mechanisms affecting short- and long-term dynamics in flooding-prone ecosystems.

**Key words:** Secondary roots, aerenchyma, genotypic variation, petiole length, plant performance, root porosity, selection, soil flooding, specific leaf area (SLA), *Trifolium repens*, white clover.

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INTRODUCTION

River floodplains comprise a wide variety of habitats, but most of these habitats share flooding as a common disturbance factor. Flooding may occur for short or longer periods during the growing season, but invariably results in strong effects on standing biomass of the local terrestrial vegetation. This has resulted in numerous publications that have studied the responses of river foreland and other plant species, and which have often contrasted species from higher with those from lower elevated sites (e.g. Justin and Armstrong, 1987; Laan et al., 1989; Blom et al., 1994; Mommer et al., 2005). Flooding is characterized by oxygen-deficient conditions (Jackson and Colmer, 2005), and previous studies have, therefore, particularly focused on variation between species in metabolism related to anaerobic respiration, and on variation in morphological and anatomical characteristics, such as shoot elongation (reviewed in Voesenek et al., 2006), leaf heterophyll (Mommer and Visser, 2005), adventitious or secondary roots and aerenchyma (Visser and Voesenek, 2005), which all aid in restoring the supply of oxygen to the submerged plant tissues.

It is likely that not only do species vary in their tolerance and response to flooding, but that also within-species differences will frequently occur. Several studies on inter-population differences have used populations from contrasting sites. Experiments on tupelo (*Nyssa sylvatica s.l.*) indicated that seedlings from populations in floodplains were much more flooding resistant than those from upland populations, but less resistant than seedlings derived from swamp populations (Keeley, 1979). Similarly, *Ranunculus reptans* plants from lower areas in a floodplain showed higher fitness during flooded conditions than plants collected at nearby but higher elevation and thus less frequently flooded sites (Lenssen et al., 2004). Other studies also support this view that, in general, populations from flooding-prone habitats appear more resilient to experimental flooding and/or show more extensive morphological and physiological responses when flooded (e.g. *Ranunculus repens*, Lynn and Waldren, 2003; *Veronica peregrina*, Linhart and Baker, 1973). This...
suggests that, in an evolutionary context, selection has taken place for traits favouring flooding tolerance.

Surprisingly, most of the studies mentioned above have not attempted to link genotypic variation in the expression of ecophysiological and morphological traits commonly assumed to increase performance during flooded conditions to actual plant performance in response to flooding. One exception are the negative correlations found by Lenssen et al. (2004) between both rosette size and internode length and fitness during flooding for 18 genotypes of the clonal species *R. reptans*. In another study, 5 d of soil flooding resulted in a strong positive correlation between activity of alcohol dehydrogenase (an enzyme involved in anaerobic respiration) and relative growth rate in clones of white clover (*Trifolium repens*) from three populations (Chan and Burton, 1992).

Such approaches yield valuable information on whether the traits under investigation are potentially under selective pressure, and may reveal how variation in these traits translates into performance of the plant during stress conditions. The presence of within-population variation in flooding tolerance-related traits indicates that there is still potential for future selection on these traits (Mitchell-Olids and Schmitt, 2006; Barrett and Schlutter, 2008). The aim here is therefore to show, based on an experimental approach, that within-population variation in the expression of flooding-related traits differentially affects performance of individual genotypes in flooded and non-flooded conditions.

An experiment was designed in which the constitutive and inducible expression of selected traits of genotypes from a single population was linked to the performance of these individual genotypes when challenged with flooding.

Recent work by our group has shown that white clover plants originating from the same population can have a wide variety of expression of traits such as petiole length and leaf size (Weijschedé et al., 2006, 2008a, b). This makes the species an excellent tool for studying the effects of trait variation on plant performance over a wide range of potential environmental factors. The fact that it is a clonal species simplifies experimentation, as individuals of the same genotypic background can easily be used as replicates in experimental set-ups. White clover is a common species along European river forelands, and it is likely that traits that are beneficial for counteracting the adverse effects of flooding are, at least to a certain extent, present in these floodplain populations. Chan and Burton (1992) studied variation in anaerobic metabolism in this species, but morphological responses such as secondary root formation and aerenchyma development are likely to be more beneficial during longer-term soil flooding, because of their contribution to better aeration of the plant and thereby to a more efficient carbohydrate metabolism. It is well known that morphological acclimation to flooding takes place in *Trifolium* species, as shown by, amongst others, Rogers and West (1993), Gibberd et al. (1999, 2001) and Aschi-Smiti et al. (2003). Therefore, the present study focuses on morphological traits of white clover rather than on its metabolical characteristics.

We were particularly interested in whether we could find variation for flooding tolerance-related morphological traits such as secondary root formation and aerenchyma content between floodplain white clover genotypes, and whether this variation was sufficiently high to have effects on the fitness of these genotypes during a period of shallow flooding. A range of genotypes of this species were therefore selected to show that (1) constitutive expression of such traits in common non-flooded conditions varies considerably, (2) the morphological responses to partial submergence also differ widely among genotypes, and, moreover, (3) plastic and constitutive variation in trait expression affect performance in soil-flooded plants.

**MATERIAL AND METHODS**

**Plant species**

The species *Trifolium repens* L. was used for the experiment. *Trifolium repens* is a common species occurring in numerous habitats ranging from pastures, road side verges to frequently flooded, riverine habitats. This species is characterized by horizontal, above-ground stolons on which the ramets are situated. Each ramet consists of an internode, one leaf, root primordia and one axillary meristem situated in the leaf axil. New ramets can be produced by either growth of the apical meristem or by induction of new branches emerging from axillary meristems (Huber and During, 2000; Newton and Hay, 1996). Two root primordia are present at each node, which give rise to the primary roots. However, plants have the capacity to grow additional roots, here referred to as secondary roots. Although ramets are highly integrated (Turkington and Klein, 1991), individual ramets are potentially independent. Ramet production can therefore be regarded as vegetative reproduction. In *T. repens* either the number of vegetatively produced ramets or total plant biomass can be assumed to be an indicator of plant performance (Weijschedé et al., 2006).

In 2001, 107 rooted stolons of *T. repens* were collected in a riverine grassland near Ewijk (the Netherlands, 51°52′54″N, 5°45′00″E). After collection, the plant material was maintained in common-garden conditions at Radboud University Nijmegen for several years to reduce potential carry-over effects. Molecular techniques [amplified fragment length polymorphism (AFLP), four primer combinations, 145 markers] were used to establish the genetic identity of the plants (J. Peeters, Radboud University Nijmegen, Nijmegen, the Netherlands, unpubl. res.). From the resulting 87 genotypes distinguished by AFLP, a subset of morphologically contrasting genotypes were chosen for further analyses (Weijschedé et al., 2006, 2008a, b), from which 18 genotypes were selected and propagated to be used in this experiment.

In autumn, 2004, these 18 genotypes were transferred to a heated greenhouse and planted in individual trays filled with a 1 : 1 mixture of sand and sieved potting compost. Plants were fertilized with 100 mL half-strength Hoagland solution twice (Hoagland and Arnon, 1950) during propagation. The plants were maintained in the same greenhouse throughout propagation and the actual experiment. Light was supplemented by high-pressure sodium lamps (SON-T, 600 W, Philips, Eindhoven, the Netherlands) whenever light intensity decreased below 350 W m$^{-2}$ between 0700 and 1100 h. Starting in February, 2005, trays (length × breadth × height: 22 × 12.5 × 5 cm) were filled with sieved loamy sand 2 weeks prior to planting, with 12 g of slow-release fertilizer...
(Osmocote Mini Exact, NPK 16 + 8 + 11 + 2MgO + trace elements, release time 3–4 months, Scotts International, Geldermalsen, the Netherlands) added per 5 L of substrate.

**Experimental set-up**

At planting, two lateral cuttings were taken per genotype. Each cutting was a fully grown rooted ramet with an initiated axillary branch consisting of three to five ramets. Cuttings were carefully planted into separate trays and pinned down to the substrate with plastic-coated wire to ensure good contact with the soil. In order to facilitate establishment further and to ensure sufficient air humidity to induce rooting, the trays were covered with transparent plastic for 3 d. Planting was repeated in four temporal blocks with a 1-week interval between blocks. Two weeks after planting, the treatments were started. A total of 144 plants were used in the experiment (18 genotypes × 2 treatments × 4 temporal blocks).

In order to test for the effects of waterlogging on plant performance two water treatments were applied. In the control (drained) treatment, plants were watered three times a week until soil water percentage reached 25% (v/v, as measured with a ThetaProbe Soil Moisture Sensor, Delta-T Devices Ltd, Cambridge, UK). Pilot experiments had shown that at a soil water percentage of 25% the soil was almost at field capacity and plant growth was optimal. In the soil flooding treatment, trays were placed into bigger, water-filled trays. The water level was kept constant at 1–3 cm above the substrate. In the soil flooding treatments the stolons, younger leaves and roots were submerged and only petioles, mature leaf blades and flowers could protrude above the water surface.

Treatments were continued for 4 weeks after which the plants (consisting of the basal portion present at the onset of treatments and the newly formed ramets) were harvested. At harvest, the roots were carefully washed free of soil. To be able to perform root porosity measurements, the plants were rinsed with special care in order to prevent the roots from bruising. After washing, the plants were put in sealed plastic bags with the roots packed in wet paper tissue in order to prevent them from losing turgor, and kept in a refrigerator at 4 °C for a maximum of 24 h.

Root porosity was determined using the microbalance method as described in Visser and Bögemann (2003). Essentially, 10–15-mm-long sections were taken from the base of roots that grew from the nodes of the stolon. We aimed at sampling those parts of the root system that were, under the given conditions, supporting growth of the ramet. Therefore, in case of the soil-flooded plants, newly formed secondary roots were taken that developed during the treatment, whereas in non-flooded plants the single, primary roots that developed at most of the nodes were used. After cutting these sections with a razor blade, the segments were gently blotted with dry tissue paper, placed in a gelatine capsule (type Helder-2; Bloklandpack BV, IJsselstein, the Netherlands) of known weight, and weighed on a microbalance (Sartorius Micro M2P). Air was then removed from the tissue by vacuum infiltration under water, after which the segments were weighed again. The volume of the internal air space could then be obtained from the difference between these two weight values, whereas the volume of the total segment was calculated from the weight of the infiltrated tissue and the specific weight of this type of tissue, which had been determined on a larger sample of root tissue (1.037 g ml⁻¹; for details on this method see Visser and Bögemann, 2003). Finally, root porosity was calculated as the percentage of air space per volume of tissue. Some samples were used to make free-hand cross-sections and studied under a microscope to determine the type of aerenchyma formation.

After sampling for root porosity measurements, the plants were divided into roots, stolon-internodes, leaves (petioles and lamina) and flowers. As the plants were maintained in a heated greenhouse during winter and the experiment was performed in early spring, only few of the genotypes produced flowers, and flowers were therefore discarded in our further analysis, but included in total dry weight increment. The number of ramets on the main stolon, the number of lateral branches, the total number of ramets and the number of secondary roots on the primary stolon were counted. In order to get information about specific trait values expressed in the two treatments, the 3rd youngest (and unrooted) ramet was measured separately. For this ramet, porosity of the preceding internode, the petiole length and leaf area, as well as dry weight of these organs was determined individually. Stolon internode porosity was measured similar to the methods described for root porosity. For these measurements, a piece of the internode of 1–1.5 cm was dissected between the third and fourth node. Care was taken not to cut too close to the nodes as the morphological structure of internodes is different from the nodes. Dry weights were measured after drying plant parts for at least 48 h at 70 °C to constant weight. Specific leaf area (SLA) was calculated by dividing leaf area by dry weight of the leaf. The third youngest ramet was used in order to be able to compare trait values expressed at the same developmental stage. Developmentally older ramets could not be used as in some conditions and in some genotypes leaf decay may already start as early as at the 4th to 5th ramet.

**Statistical analyses**

Mixed-model ANOVA was used to test for the effects of treatments, genotypes and their interaction on trait values. Treatments were considered fixed effects and genotypes as random. The random factor block was added to the model to account for differences among the four temporal blocks. For the two parameters indicating plant performance (ramet number and total plant dry weight) a mixed-model ANCOVA was performed. Ramet number at the onset of treatments was added to the model to account for variation in the initial size, which is very likely to translate into differences in the total growth achieved during the experiment.

Genotypic selection analyses were used to test for the effects of trait values in a specific environment on total plant biomass at harvest. For ramet number as an indicator for plant performance the results were largely comparable. In addition, a significant difference among genotypes was found only for biomass production. Therefore, we decided to concentrate on total plant biomass for the remaining analysis. We calculated selection gradients (β), which are the partial
regression coefficients of relative performance on a trait value, and which are a measure of the degree to which change in the value of a trait is associated with a change in the relative fitness of individuals (Wade and Kalisz, 1989). A positive value for the selection gradient indicates that an increase in the trait value is associated with relatively higher plant performance while a negative value for the selection gradient indicates that an increased trait value is associated with relatively lower plant performance. Positive selection gradients can therefore be interpreted to lead to selection for higher trait values (e.g. increased root porosity), while negative selection gradients will lead to selection for lower trait values (e.g. decreased root porosity). As environmentally induced covariances between trait values and performance can lead to biased estimates of selection, genotypic means rather than phenotypic values of individual plants were used to calculate selection gradients (Stinchcombe et al., 2002; Mauricio and Mojonnier, 1997). Genotypic relative performance was calculated by dividing genotypic means within treatment by the overall treatment mean. Trait values were relativized by subtracting the overall mean from the genotypic mean of a trait and dividing it by the overall standard deviation of a trait among genotypes. This allows for direct comparison of the selection gradients among traits and treatments.

Path analyses were used to investigate the effects of trait values in control conditions and the plasticity of traits in response to soil flooding on the change of biomass in response to flooding. Relative plasticity and biomass change were calculated from the difference in the genotypic mean trait value (soil-flooded – control conditions) divided by the value expressed in control conditions. A negative value indicates a reduction of a trait value or reduced performance of a given genotype in soil-flooded relative to control conditions while a positive value would indicate either that a trait value gets larger or that the relative performance of a genotype is higher in soil-flooded as compared with control conditions. Relative plasticity was calculated in order to be able to compare smaller and larger genotypes (Huber, 1996). In the path analyses we tested for the effects of the constitutive trait value expressed in control conditions on the soil-flooding-induced changes of the trait values (i.e. their plasticity). A significant effect of constitutive value on its plasticity would indicate that the plasticity of a trait value depends on the value expressed in control conditions. It would be positive if genotypes with a larger trait value were also characterized by higher plasticity. A negative effect of constitutive value on plasticity indicates that genotypes with larger trait values are less plastic, or even reduce their trait values in response to flooding.

We then tested how a constitutive trait value and its plasticity affect the change of biomass production in soil-flooded as compared with control conditions. A positive effect indicates that genotypes characterized by either a large trait value or a large trait value plasticity suffer relatively less from soil flooding or even show increased performance than genotypes with lower trait values or lower plasticity, respectively. The program package Amos (Arbuckle and Wothke, 1999) was used for the path analyses.

### RESULTS

**Responses of plants to soil flooding**

Plant performance was significantly reduced by soil flooding (Table 1). The total number of ramets at the end of the experiment was reduced by 36%, while total dry weight was reduced by 17%. There was significant variation among genotypes for total plant dry weight, but not for ramet number.

Porosity in the basal parts of the roots in control conditions was relatively low, i.e. around 6% (Table 1). Soil flooding, however, significantly increased root porosity, to, on average, 60% higher levels. Root porosity tended to differ among genotypes and the individual genotypes responded significantly differently to soil flooding (Table 1). Most of the genotypes strongly increased root porosity when flooded, while some genotypes were characterized by relatively constant root porosity over the two treatments (Fig. 1; treatment × genotype interaction in Table 1). Soil flooding also induced secondary root formation at the nodes along the primary stolon (Table 1). In control conditions plants hardly produced any of these roots.

### Table 1. Responses of plants to treatments and the results of a mixed-model ANOVA testing for the effects of treatments (fixed effect) and genotypes (random effect) on plant traits and performance

<table>
<thead>
<tr>
<th>Traits</th>
<th>Control</th>
<th>Soil flooding</th>
<th>Treatment (1)</th>
<th>Genotype (17)</th>
<th>Treatment × Genotype (17)</th>
<th>Block (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root porosity</td>
<td>5.7 ± 0.5</td>
<td>9.1 ± 0.8</td>
<td>11.57**</td>
<td>1.55$</td>
<td>1.82*</td>
<td>8.32***</td>
</tr>
<tr>
<td>Secondary roots per primary node</td>
<td>0.03 ± 0.01</td>
<td>1.30 ± 0.09</td>
<td>200.83***</td>
<td>1.07 n.s.</td>
<td>0.95 n.s.</td>
<td>3.60*</td>
</tr>
<tr>
<td>Petiole length</td>
<td>64 ± 3</td>
<td>79 ± 4</td>
<td>14.17**</td>
<td>3.52***</td>
<td>0.90 n.s.</td>
<td>179.11***</td>
</tr>
<tr>
<td>Leaf area</td>
<td>5.69 ± 0.36</td>
<td>7.19 ± 0.40</td>
<td>14.89**</td>
<td>5.56***</td>
<td>1.86*</td>
<td>0.14 n.s.</td>
</tr>
<tr>
<td>Specific leaf area</td>
<td>39.1 ± 2.2</td>
<td>43.2 ± 1.3</td>
<td>7.27*</td>
<td>2.86***</td>
<td>1.11 n.s.</td>
<td>6.03***</td>
</tr>
<tr>
<td>Percentage allocation to leaves</td>
<td>54.2 ± 0.6</td>
<td>51.4 ± 1.0</td>
<td>11.97**</td>
<td>4.01***</td>
<td>1.53 n.s.</td>
<td>3.21*</td>
</tr>
<tr>
<td>Total ramet number</td>
<td>57.5 ± 3.5</td>
<td>36.6 ± 2.4</td>
<td>54.23***</td>
<td>1.21 n.s.</td>
<td>0.72 n.s.</td>
<td>1.50 n.s.</td>
</tr>
<tr>
<td>Total plant dry weight</td>
<td>1.03 ± 0.05</td>
<td>0.86 ± 0.05</td>
<td>14.31**</td>
<td>2.06*</td>
<td>0.59 n.s.</td>
<td>1.05 n.s.</td>
</tr>
</tbody>
</table>

Means are given ± s.e. For the two growth-related performance parameters we added initial ramet number measured at the onset of treatments as a covariable to the model to account for differences in initial plant size. Initial ramet number significantly affected final ramet number and biomass (F-values and their significance of the effects of initial ramet number were 46.99*** for total ramet number and 55.83*** for biomass). Treatment means and standard errors were calculated by using genotypic means. F-values and their significance are given. Significance levels are as follows: n.s.: $P > 0.10$; $^5$: 0.05 < $P < 0.10$; $^*$: 0.01 < $P < 0.05$; $^{**}$: 0.001 < $P < 0.01$; $^{***}$: $P < 0.001$. 

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but relied on primary roots, while in soil-flooded conditions many of the nodes produced at least one secondary root. For this trait, there was no significant difference among genotypes and in the response of genotypes to soil flooding (Table 1, Fig. 1).

The porosity of stolon internodes of plants subjected to control conditions was 8.0% (± 0.4) and in waterlogged plants 7.3% (± 0.6). There was neither an effect of genotypes or treatments on the porosity of internodes nor an effect of the porosity of internodes on plant performance (data not shown), indicating that stolon aerenchyma formation is not variable and does not contribute to adaptation to soil flooding in *T. repens*.

All leaf-related traits responded significantly to treatments (Table 1). Petiole length increased by 20%, leaf area by 26% and SLA by 10% in response to soil flooding, while the relative allocation to leaves decreased by 5%. Mean trait values differed significantly among genotypes, with the relative variation among genotypes being much larger in petiole length, leaf area and SLA than for the relative allocation to leaves (Table 1, Fig. 1). However, except for leaf area, the genotypes did not differ significantly in response to soil flooding (Table 1).

Plasticity of trait values strongly depended on the constitutive trait value expressed in control conditions (vertical...
arrows in Fig. 2). For the traits root porosity, petiole length, SLA and leaf area, genotypes displaying high trait values in control conditions were less plastic in response to soil flooding than genotypes with lower trait values in control conditions. This means that having a high porosity, petiole length, leaf area or SLA in control conditions resulted in less change in these traits during soil flooding. The flooding-induced plasticity in secondary root formation, by contrast, was positively associated with the number of secondary roots produced in control conditions (Fig. 2).

**Effects of trait values on plant performance**

In control, drained conditions none of the root-related trait values affected biomass production. For the leaf traits only SLA had an effect. Apparently, genotypes characterized by high SLA performed relatively better than genotypes with lower SLA (Table 2). In soil-flooded conditions, root porosity and petiole length were significantly positively associated with dry weight. In flooded conditions, genotypes with either higher root porosity or longer petioles had a higher dry weight at harvest.

One of the aims of this experiment was to investigate how constitutive trait values and flooding-induced trait value plasticity affect changes of performance in soil-flooded as compared with control conditions. In this regard, we tested for the effects of specific trait values in drained conditions and their plasticity on change of biomass in soil-flooded conditions relative to the control conditions. Of the six traits tested, only root porosity, SLA and production of secondary roots significantly affected flooding-induced changes of biomass production (Fig. 2). By contrast, petiole length, leaf area and leaf weight ratio and their plasticity were not associated with flooding-induced changes in biomass production. Root porosity and, in particular, the flooding-induced increase of root porosity had a positive effect on plant performance, although absolute changes in porosity were not large. This means that genotypes characterized by highly porous roots suffered less from soil flooding than genotypes characterized by less porous roots. Similarly, genotypes producing secondary roots in control conditions tended to be less affected in biomass during flooding than genotypes producing fewer or no roots in control conditions. However, the flooding-induced increase of the number of secondary roots was, surprisingly, associated with decreased performance. Similarly, high SLA and flooding-induced increases in SLA were both associated with relatively poorer performance. Biomass production of genotypes with high SLA (presumably producing thinner leaves) in control conditions was more vulnerable to soil flooding than that of genotypes characterized by low SLA (and therefore relatively thicker leaves). Genotypes responding to soil flooding by having higher SLA also tended to grow less well during soil flooding than genotypes being less responsive in SLA.

![Diagrams showing path analyses](https://academic.oup.com/aob/article-abstract/103/2/377/187734)
Environmental variation is a pervasive feature of virtually all ecosystems, and organisms have the potential to adapt to variable conditions at different ecological and evolutionary scales (Stuefer, 1996; Stratton and Bennington, 1998; Zhang, 2005; Zhang and Hill, 2005). In general, adaptive evolutionary change relies on the presence of molecular and phenotypic variation in a population and the relation between trait expression and fitness on which selection can act (Via and Lande, 1985; Gomulkiewicz and Kirkpatrick, 1992; Huber et al., 2004; Kingsolver and Pfennig, 2007). Whether selection acts on specific plant characteristics depends on the predictability and strength of selection pressures (Wade and Kalisz, 2000; Volis et al., 2002; Huber et al., 2004).

In riverine grasslands, gradients from the river to higher elevated sites feature distinctly different conditions at their extremes, with high levels of physical disturbance caused by flooding close to the river, and high levels of competition and a higher likelihood of water shortage at higher elevations (Lenssen and de Kroon, 2005). Physical disturbance and inundation stress due to repeated flooding exerts strong selection pressures on plant species, resulting in a conspicuous zonation of plant species displaying different degrees of adaptations to flooding, depending on their position along the elevation gradient (Blom et al., 1990; van Eck et al., 2004; Voesenek et al., 2004). Although these among-species differences in traits allowing plants to survive in flooded conditions are well understood (Visser and Voesenek, 2005; Bailey-Serres and Voesenek, 2008), species adapted to flooding generally have relatively high root porosity and the ability to produce flooding-induced secondary or adventitious roots, both of which lead to improved oxygen status of soil-flooded plants (Visser et al., 1996, 1997). Flooding-induced petiole or stem elongation may lead to the positioning of leaves above the water surface when the floodwater level is shallow (Voesenek et al., 2004). Frequent or predictable shallow flooding is therefore hypothesized to select for high root porosity, increased secondary root formation and a high degree of petiole and stem elongation, as these adaptations are associated with increased performance of plants in flooded conditions. The fact that species growing at different positions along flooding gradients differ in the expression of these traits (Visser et al., 1996; Voesenek et al., 2004) supports this assumption. The present results show that even within species considerable genetic variation in these traits does exist.

**Genotypes of *T. repens* originating from riverine grasslands differed in their constitutive expression of root porosity.** In addition, genotypes differed significantly in their flooding-induced changes of root porosity, ranging from an up to three-fold increase in root porosity by some genotypes to a 50% decrease in root porosity by other genotypes. Variation in root porosity was linked to performance differences in flooded but not in drained conditions. Both high levels of constitutive root porosity and plasticity of root porosity when exposed to flooding positively affected relative performance in flooded as compared with drained conditions, indicating that different genotypes were selected for in flooded and drained conditions. Genotypes with relatively high levels of root porosity were hardly affected by flooded conditions, whereas genotypes with low levels of root porosity suffered significant biomass reduction compared with drained conditions. These results clearly support the notion that within-species variation in root porosity is present in populations growing in environments prone to flooding and can lead to shifts in the relative frequency of genotypes depending on the frequency of flooding. The maximum root porosity observed in our experiment (i.e., below 12%, except for one genotype) does not classify white clover as having particularly strong aerenchyma formation, as typical wetland species may have root porosities in excess of 30% (Justin and Armstrong, 1987). However, it is particularly in this lower
range that one may expect the strongest effects of changes in porosity on internal aeration (Armstrong, 1979; van Noordwijk and Brouwer, 1993), which explains why even a relatively low variation in root porosity led to differential growth among genotypes subjected to soil flooding.

In contrast to previous results on other species (Laan et al., 1989; Visser et al., 1996; Garthwaite et al., 2003), we found only a relatively small number of flooding-induced secondary roots in white clover. The number of secondary roots also hardly affected plant growth responses to flooding, with plastic increases in the number of secondary roots even negatively affecting biomass production. These results may be explained by the architecture of *T. repens*. This plant grows by means of above-ground stolons and produces a new primary root at each stolon node from the root primordia that touches the moist soil (Thomas et al., 2002; Thomas and Hay, 2008). These roots have the potential to grow superficially instead of deeper in the soil when flooded, thereby avoiding oxygen-deficient soil layers (as described by Gibberd et al., 2001; and similar to the horizontally growing roots in *Rumex* species shown by Laan et al., 1989). Many of these primary roots did not penetrate the soil surface at all but developed into well-branched root systems in the shallow water layer. Additional secondary root formation may then not give a further advantage to the plant, and can thus be expected to be selectively neutral. Increased flooding frequency and depth are then unlikely to result in selection for increased secondary root production.

If plants are completely submerged, increased petiole and stem length is widely assumed to increase performance (Voesenek et al., 2006; Jackson, 2008). Even in our experiment, where plants were not completely submerged, flooded plants increased their petiole length by on average 15% and genotypes with longer petioles had a significantly higher biomass production in flooded but not in drained conditions. However, neither constitutive petiole length nor petiole length plasticity were associated with differences in biomass production in flooded as compared with drained conditions (see Fig. 2). Increased petiole length may help young leaves, which developed at the stolon apex and were initially under water, to reach the water surface earlier and ensure contact with the air earlier in the sensitive and important stage of early leaf development. This indicates that whereas in drained conditions petiole length appears to be selectively neutral, increased petiole length may be selected in flooded conditions (see Table 2). As a result, overall petiole length can be expected to increase gradually in regularly flooded environments.

In response to soil flooding, plants produced larger and thinner leaves, which somewhat resembles the changes of leaf traits in completely submerged conditions (Mommer et al., 2005). In the present experiment the relative allocation to leaves was slightly reduced in flooded conditions, which is in contrast to the expectation that flooded plants should increase investment into the leaves at the cost of roots. Neither leaf area nor relative allocation to leaves directly affected performance of plants subjected to flooded or drained conditions or the relative response of plants to flooding. SLA, however, strongly contributed to variation in plant growth. Although plants tended to benefit from the production of thinner leaves in drained conditions (Table 2), the production of thinner leaves negatively affected the relative response of plants to soil flooding (Fig. 2), indicating that opposite selection pressures acted on plants depending on soil water status. Whereas in drained conditions genotypes with thin leaves were able to produce more biomass, the same genotypes were selected against during soil flooding. In addition, genotypes expressing higher degree of flooding-induced plasticity in SLA were selected against. Although high SLA has been shown to increase the capacity for gas exchange with the surrounding water when plants are completely submerged (Mommer et al., 2005), it is unclear why this trait would be disadvantageous during soil-flooded conditions. Potentially, water loss due to evapotranspiration might not be matched by sufficient water uptake due to anoxia in the root system, but given the shallow rooting of *T. repens*, even partly in the floodwater layer above the soil, this is unlikely to be an explanation in our experimental system.

CONCLUSIONS

Environmental conditions in riverine grasslands of low elevation change frequently during the growing season of plants, as flooding may occur at any time during the year. Although the exact timing of flooding is unpredictable, the fact is that flooding will occur at some stage during the lifetime of a plant. Long-lived clonal species, such as *T. repens*, will thus repeatedly be subjected to flooding, which therefore constitutes an important selection pressure in this ecosystem. The present data clearly show that experimental shallow flooding selected for high root porosity and stronger petiole elongation, and that the specific selection pressures exerted by flooding are very different from those in drained conditions. Different genotypes, characterized by their own, unique combination of traits, will proliferate well in some conditions, but not equally well in others. This temporal variation in selection pressures will, in combination with spatial variation in micro-habitat conditions caused by, for example, slight and small-scale differences in elevation, contribute to the maintenance of genetic diversity in the dynamic system of riverine grasslands. These grasslands thus represent an interesting setting to study the impact of evolutionary processes exerted by temporal and spatial micro-habitat variation on plant characteristics. Understanding the interaction between micro-habitat conditions, plant characteristics and plant performance will enable us to predict the mechanisms affecting short- and long-term dynamics of plant species and their specific adaptations in this system.

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