Influence of Position and Number of Nodal Roots on Outgrowth of Axillary Buds and Development of Branches in *Trifolium repens* (L.)

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The implications of the presence of a root, either at the parent node or at neighbour nodes, on branch formation of *Trifolium repens* (white clover) was investigated. Plants were freely rooted or rooting was restricted to every sixth or every twelfth node along the parent axis. The absence of a root at the parent node had little influence on the probability of the subtending axillary bud forming a branch but, on average, delayed the outgrowth of the bud. The probability that an axillary bud, emerging from a non-rooted parent node, developed to a lateral branch (branch with elongated internodes) decreased with decreasing proximity of the parent node to a rooted node. Lateral branches emerging from non-rooted parent nodes which were two nodes distal to a rooted node had a higher rate of node appearance, a greater mean internode length and area per leaf, and were more branched than lateral branches emerging from other non-rooted parent nodes. The dry mass of each single root and of branches grown at rooted parent nodes were significantly higher in plants with restricted rooting than in freely rooted plants. Restriction in the number of rooted nodes per plant increased the number of inflorescences. It is concluded that the whole plant response to restricted root formation was continuous growth of the parent axis and compensatory growth of the branch at the rooted node. In general, growth was slow for axillary buds whose development was dependent on the basipetal movement or cross-transport within the stolons of resources exported from roots.

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INTRODUCTION

The persistence and spread of white clover (*Trifolium repens* L.) depend largely on its ability to branch. In order to understand seasonal fluctuations in yield of *T. repens*-based pastures processes involved in branching need further clarification. The formation of a shoot branch comprises four successive stages: (*a*) the initiation and development of the axillary bud prior to outgrowth, (*b*) outgrowth of the axillary bud, (*c*) development of the branch and (*d*) establishment of a branch which involves achieving a positive carbohydrate balance and the formation of roots. Each process may be influenced by several factors. For example, both defoliation (Davies and Evans, 1990) and additional far-red light (Robin *et al.*, 1994) delayed outgrowth of axillary buds in *T. repens*. Many clonal species have higher rates of branching in favourable patches, i.e. patches with high light availability and mineral nutrients (de Kroon and Hutchings, 1995). Therefore, in plant growth models branching is considered a key property which enables clonal plants to forage in a patchy environment (Sutherland and Stillman, 1988; Cain, Dudle and Evans, 1996). However there is some evidence that branching might not only be influenced by resource supply but also by the presence of a root at a node. For example, Chapman (1983) found that when a root was present at the parent node a branch developed faster and survived longer than when a

root was absent. However, among branches developing from non-rooted parent nodes little distinction has been made between their origin relative to the nearest rooted node. Absence of a root at a node on relatively short stolon sections which had only a few nodes did not inhibit the outgrowth of the adjacent axillary bud (Davies and Evans, 1990; Jones and Sackville Hamilton, 1993). On the other hand, in plants which were only rooted at the base, outgrowth of axillary buds was suppressed on long non-rooted stolons (Thomas, 1987*a*). These findings suggest that the number of successive non-rooted nodes along a stolon may be a factor influencing the outgrowth of axillary buds. Bud outgrowth and development and establishment of branches may also depend on the position of the parent node relative to the nearest rooted node. Relatively weak correlations between branching and rooting frequency (Chapman, 1983) indicate that some of the branches emerging from non-rooted parent nodes may also have a high probability of establishment.

Frequency of presence of a root at a node in field grown plants is, on average, below 50 % and sometimes less than 20 % (Chapman, 1983; Newton and Hay, 1994). Axillary buds, therefore, occur more frequently on non-rooted than on rooted nodes. The rooting pattern of a *T. repens* plant may influence the fate of an axillary bud in two ways: firstly, by the number of rooted nodes per plant. Such a relationship would reflect processes which control plant development as a unique, integrated system. Secondly, by the position of the parent node relative to the nearest rooted node. Such a

response would be a local effect. The aim of this study was to investigate whether variation in rooting pattern influences branching of *T. repens* by whole plant and/or local responses. Plants were established with differing sequences of rooted nodes, and as a consequence, plants had different numbers of rooted nodes. Thus, the influence of whole plant responses on the fate of axillary buds were tested by comparing plants of different rooting patterns, whereas local responses were tested by comparing, within plants, the fate of axillary buds which had parent nodes at differing positions relative to a rooted node.

MATERIALS AND METHODS

Definitions

Vegetative outgrowth of a shoot at a node was defined as a branch. Outgrowth was defined as having occurred when the first leaf of the axillary bud was visible beyond the stipule at a node and greater than 0·5 on the Carlson scale (Carlson, 1966). The time until a branch appeared was the number of days between a subtending leaf achieving the 0·5 stage of development and the first leaf of its associated branch attaining the same stage of development. Branches on the parent axis were classified according to their development (Fig. 1). Branches were defined as axillary branches when there was no elongation of internodes whereas lateral branches had elongated internodes. With increasing hierarchy of branching, branches were classified as primary, secondary or tertiary.

Plant culture

Plants were cloned by taking cuttings from a single large-leaved genotype of *T. repens* cv. Ladino. The cuttings were planted in quartz sand in order to trigger formation of roots. After 4 weeks, single plants were transplanted into a pot (0·9 l; 12 cm diameter) filled with three parts of a clay-soil and one part of quartz sand (0·7–1·2 mm particle size). The plants were placed in a glasshouse, watered daily and supplied weekly with nutrient solution (40 ml per pot) containing (mM) 7·5 N (NO_3), 0·5 P, 3·0 K, 2·5 Ca, 1·0 Mg, 1·0 S, 0·5 Na, 0·5 Cl, and (μM) 107 Fe, 23 B, 4·55 Mn, 0·38 Zn, 0·16 Cu, 0·05 Mo (pH 5·2). Four weeks later, plants which had approximately 12 nodes on the parent axis were selected for the experiment. Each pot was then placed beside a plastic container (50 cm long × 36 cm wide × 13 cm deep) filled with the soil:sand mixture mentioned above and inoculated with *Rhizobium trifolii*, strain RCR5 (Rothamsted, UK). The stolon of the parent axis of each plant, which at this stage extended beyond the edge of the original pot, was positioned and fixed with wire hoops onto the surface of an adjacent container (Fig. 2 A). Three weeks later, the parent axes were severed from the original plant in order to get uniformly rooted plants within the treatments. By this time the severed parent axis had ten nodes. Plants were watered daily and supplied weekly with nutrient solution (750 ml per container). The containers were rearranged weekly to minimize any effects of variation of light or

Axillary branch

Lateral branch

Unfolded leaves

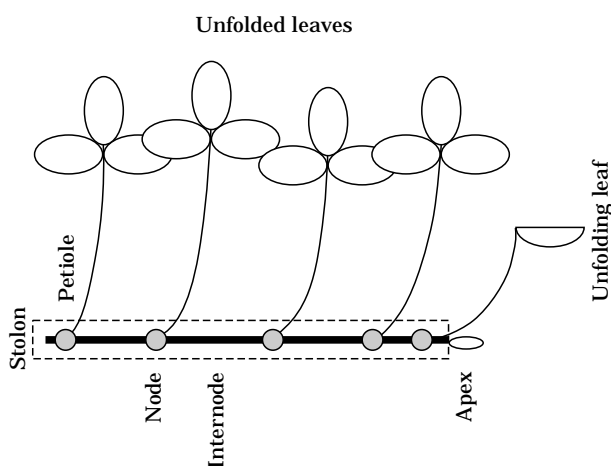Petiole
Stolon
Node
Internode
Apex
Unfolding leaf
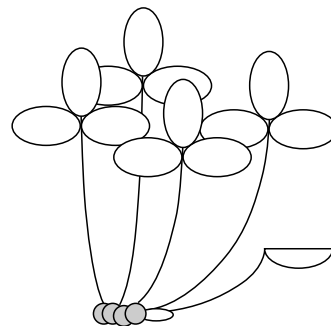
FIG. 1. Diagram of an axillary and a lateral branch.

temperature within the glasshouse. The mean day/night temperatures during the experiment (Jul.–mid Sep.) were 22·5/19·2 °C. Natural light was supplemented by high pressure vapour lamps (HPI/T 400 W, Philips) to give a daylength of 16 h (photosynthetic photon fluence rate $\geqslant 150\ \mu\mathrm{mol\ m^{-2}\ s^{-1}}$).

Treatments

Formation of roots only occurs when the relative humidity at the node is above 85 % (Stevenson and Laidlaw, 1985). Therefore the soil surface was covered with strips of plastic foil so that only chosen nodes of the parent axis came into contact with the moist soil (Fig. 2 A). In this way, three treatments were established with different sequences of rooted nodes on the parent axis (Fig. 2 B). Control plants were freely rooted (FR) along the parent axis. Plants with restricted rooting were rooted at every sixth (RR6) or at every twelfth (RR12) node of the parent axis. Plants with restricted rooting were supplied only from roots along one side of the parent axis (Fig. 2 C). There were eight replicate plants in each treatment.
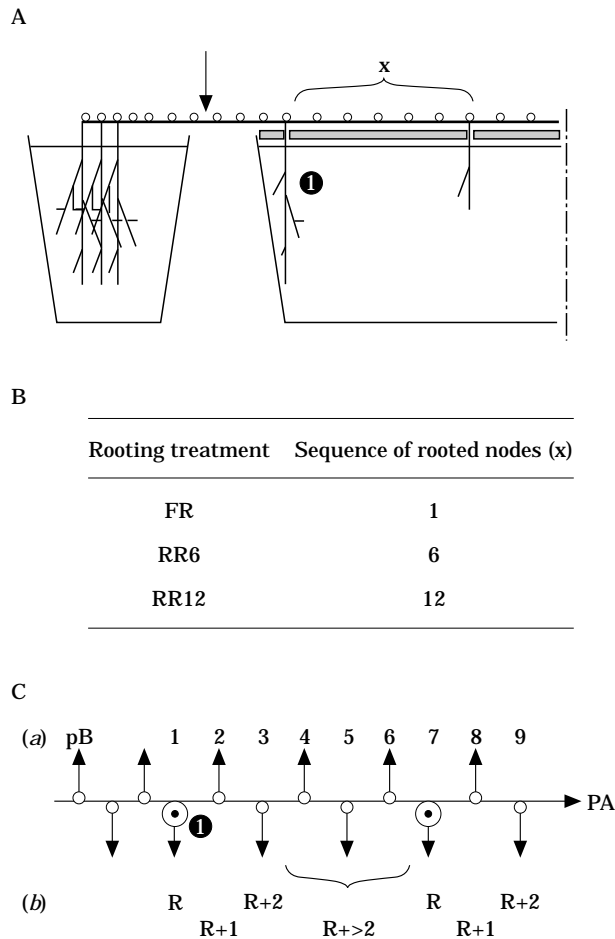
FIG. 2. A, Schematic representation of plant establishment. Cuttings of *Trifolium repens* were grown in pots. The parent axis was directed towards the surface of an adjacent container. Root outgrowth on the parent axis was triggered by fixing the chosen node onto the moist soil surface. Strips of plastic foil were laid underneath the remaining nodes. The parent axis was severed at the fourth internode (arrow) proximal to the first rooted node (❶). B, Rooting treatments applied to the parent axis. Sequence of rooted nodes (x) indicates the number of internodes present between two rooted nodes. FR, freely rooted (roots at every node); RR6, restricted rooting to every sixth node, RR12 restricted rooting to every twelfth node. C, Example of numbering of nodes according to their position along the parent axis (*a*) and according to their position relative to the nearest proximal rooted node (*b*). ⊙, rooted node (R); ◯, non-rooted node; PA, parent axis; pB, primary lateral branch. Arrows indicate direction of growth. Note the alternate orientation of the primary branches and that roots are located on the same side of the parent axis as the branches.

Measurements

The rate of node appearance on the parent axis and primary branches as well as the time until a primary branch appeared were measured. Plants were harvested 11 weeks after the parent axis was first fixed onto the soil. Each nodal root was washed out separately. The shoot was dissected into parent axis and branches. Axillary buds at nodes proximal to the youngest branched node were denoted as dormant buds. The length of each stolon was recorded and leaf area on primary branches measured (Li-3000 Portable Area Meter, LI-COR Inc, Nebraska, USA). The plant parts

were dried at 65 °C for 3 d and mass determined. In order to define the position of a primary branch along the parent axis, parent nodes were numbered in ascending order towards the apex (Fig. 2C). The position of a parent node relative to the nearest proximal rooted node was numbered beginning with R as the rooted parent node, R + 1 the next distal non-rooted parent node and so on. The relative dry mass (RDM_{ij}) of each branch (sum of primary, secondary and tertiary branches) was calculated as:

$$RDM_{ij} = DM_{ij}/DM_{iFR},$$

DM_{ij}, representing branch dry mass at the ith position of the jth treatment (RR6, RR12) and DM_{iFR}, the branch dry mass at the ith position of treatment FR. Thus, the effect of rooting can be compared between young and old branches. Branches on freely rooted plants, which emerged from non-rooted parent nodes, had a mean dry mass only 20 % of that of branches of rooted parent nodes and were not used for the calculation of RDM. The probability of lateral branch formation was calculated as the number of nodes which had a lateral branch divided by the number of nodes which had a branch (axillary or lateral branch). Only the branches at node positions 1–12 were used for this calculation because younger axillary branches had the potential to become lateral branches. Data given in tables were analysed by ANOVA (SAS Institute Inc., Cary, NC, USA) after arcsin transformation to normalize distribution where necessary. The Tukey–Kramer method was used for multiple comparisons of means.

RESULTS

When plants were allowed to root freely (FR), 77 % of the nodes along the parent axis rooted. In the restricted rooting treatments (RR6, RR12) all targeted nodes formed roots, so that at harvest RR6 plants had five and RR12 plants three rooted nodes. Rooting treatments did not influence the length (52·8 cm ± 0·80 s.e.) or number of nodes of the parent axis (32·9 ± 0·28). However, restricted rooting markedly increased the percentages of inflorescences and axillary

TABLE 1. *The influence of rooting treatments on the development of axillary buds on the parent axis of* Trifolium repens. *Data are given as the percentage (%) of total number of nodes on the parent axis. The parent axis was freely rooted (FR), rooted at every sixth node (RR6), or rooted at every twelfth node (RR12). Values with a common superscript letter within rows do not differ significantly at P = 0·05*

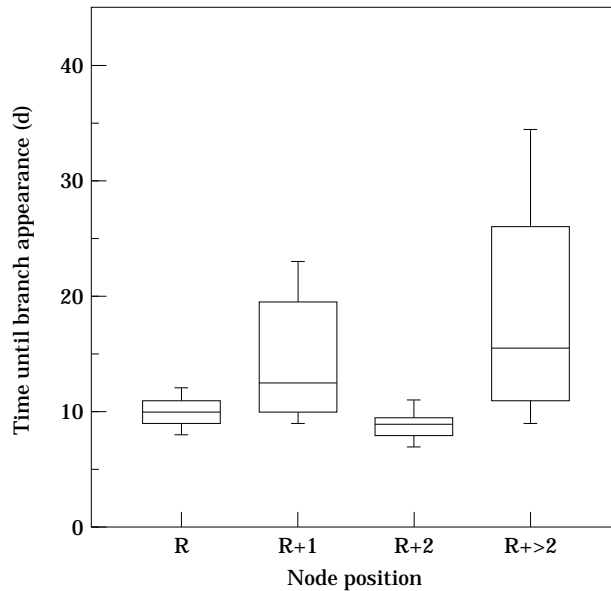|	Rooting treatments		
Developmental stage	FR	RR6	RR12
Buds distal to first branched node	13·6[a]	16·7[a]	14·0[a]
Dormant buds	1·6[b]	6·3[a]	6·7[a]
Inflorescences	0·8[c]	5·6[b]	10·8[a]
Axillary branches	14·0[c]	21·4[b]	32·9[a]
Lateral branches	70·1[a]	50·0[b]	35·7[c]

FIG. 3. Influence of nodal position of the axillary bud relative to the nearest proximal rooted node on the parent axis of *Trifolium repens* on the time until the branch appeared. R, axillary buds at rooted nodes; R+1, axillary buds at non-rooted nodes separated from the nearest proximal rooted node by one internode; R+2, axillary buds at non-rooted nodes separated from the nearest proximal rooted node by two internodes; R+ > 2, axillary buds at non-rooted nodes separated from the nearest proximal rooted node by more than two internodes. The data are given as Tukey box plots and represent 465 axillary buds of 24 plants. The line inside the box marks the value of the 50th percentile. Extents of the box indicate the 25th and 75th percentiles and bars indicate the 10th and 90th percentiles.

TABLE 2. *Influence of rooting treatments on growth characteristics of primary lateral branches of* Trifolium repens. *For rooting treatments see Fig. 2. Data of the RR6 and RR12 treatments were similar and therefore pooled. Values with a common letter within rows of the RR6/RR12 treatments do not differ significantly at* P = 0·05. *Significance of differences between rooting treatments is given for branches at position R.*

| Rooting treatments | R | Position of the parent node | | |
		R+1	R+2	R+ > 2
Node appearance rate (nodes d^{-1})				
FR	0·32			
	P < 0·001			
RR6, RR12	0·35[a]	0·28[b]	0·33[a]	0·21[c]
Internode length (mm)				
FR	13·5			
	P < 0·001			
RR6, RR12	15·5[a]	12·6[b]	14·9[a]	9·6[c]
Mean leaf area per leaf (cm²)				
FR	6·48			
	n.s.			
RR6, RR12	6·41[a]	4·20[b]	6·29[a]	3·31[b]
% branched nodes				
FR	53·4			
	P < 0·05			
RR6, RR12	66·7[a]	44·3[b]	64·6[a]	9·1[c]

Branches are grouped according to the position of their parent nodes relative to the presence of a rooted node: R, parent node rooted; R+1, parent node separated from the nearest proximal rooted node by one internode; R+2, parent node separated from the nearest proximal rooted node by two internodes; R+ > 2, parent nodes separated from the nearest proximal rooted node by more than two internodes. Each rooting treatment represents eight replicates.

branches and, to a lesser degree, the proportion of dormant buds along the parent axis (Table 1). Consequently the development of buds into lateral branches was significantly reduced when fewer nodes were rooted. All RR6 and RR12 plants, but only one FR plant, formed inflorescences on the parent axis. In all treatments first inflorescences appeared at node position 13.

The time until a branch appeared at a given node of the parent axis depended upon whether the node was rooted and also on the position of the parent node relative to the nearest proximal rooted node. At rooted nodes (R) and at the second node distal to a rooted node (R+2), branches appeared within approximately 10 d (Fig. 3). For all other buds at non-rooted nodes the time until a branch appeared varied greatly and outgrowth could occur at any time between 1 and 6 weeks. Branches emerging from rooted nodes (R) had a higher rate of node appearance, longer internodes, and a higher percentage of branched nodes in treatments RR6 and RR12 than in treatment FR (Table 2). Lateral branches at position R+2 had similar values to the branches at the rooted nodes (R). However, at positions R+1 or R+ > 2 production of nodes, organ sizes, and branching were markedly reduced.

All axillary buds at rooted nodes developed into lateral branches. The probability of a lateral branch developing was almost 100 % for the first two nodes distal to a rooted

node, but decreased greatly at nodes further from the proximal rooted node (Fig. 4). However, the probability of lateral branch formation increased at the node adjacent to a distal rooted node (R+5 in RR6 and R+11 in RR12). The effect of the position of the parent node on the dry mass of the laterals is represented by the relative dry mass (RDM$_{ij}$) at the end of the experiment. In general, lateral branches at parent nodes R+2 and R+4 had a higher RDM than branches on the opposite side (R+1 and R+3). Branch development also depended on whether the branch was distal or proximal to a rooted node. For example in RR12 (Fig. 4B), lateral branches two nodes distal to the rooted node (R+2) had a RDM ⩾ 1 whereas the RDM of branches two nodes proximal to the rooted node was < 0·5 and did not differ from the RDM of its neighbour branches. Positive effects of position (i.e. RDM > 1) occurred in young branches (positions > 12). For example, the dry mass of lateral branches at positions R, R+2, and R+4 were up to two-fold greater in the restricted than in freely rooted plants (Fig. 4). On the other hand, negative effects of position (RDM < 1) were reflected in old branches where RDM were as low as 0·1.

In plants with only a few rooted nodes (RR6, RR12), the root mass of each single root was more than twice that of
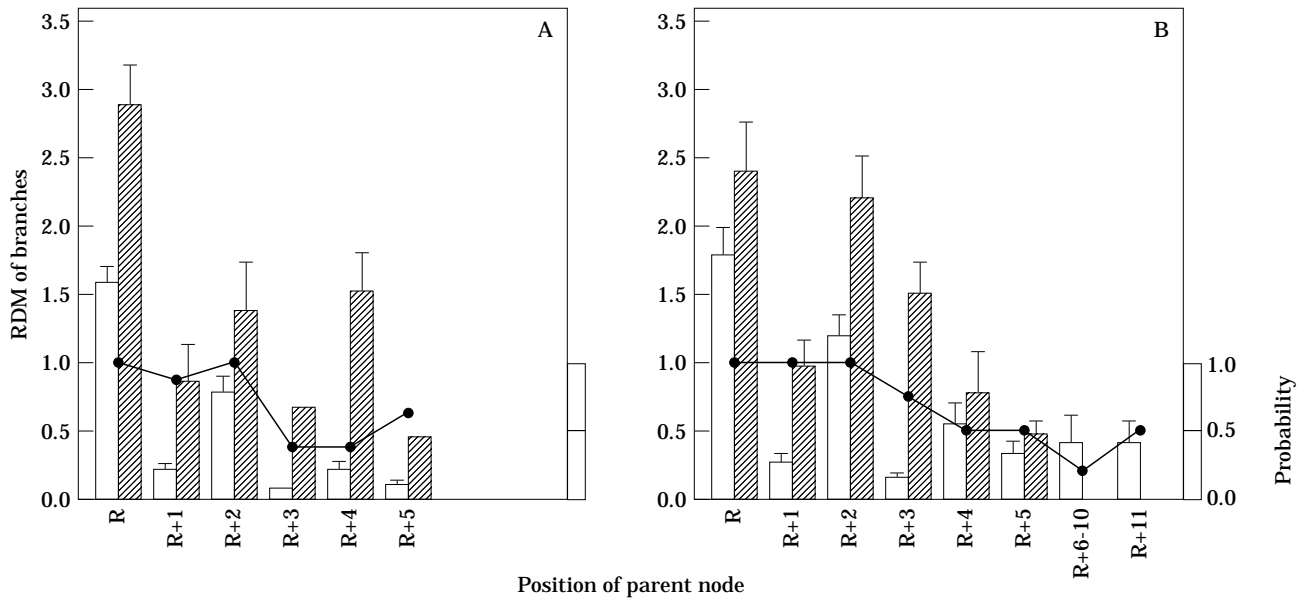
FIG. 4. Influence of the position of the parent node relative to a rooted node on the probability of lateral branch formation (●) and on the relative dry mass of lateral branches in *Trifolium repens*. Nodes on the parent axis were numbered in ascending order towards the apex. A, Plants were rooted at every sixth node. Data is presented for relative dry mass of lateral branches at node positions 1–6 (□) and 19–24 (▨). B, Plants were rooted at every twelfth node. Lateral branches were at node positions 1–12 (□) and 13–24 (▨). There were no lateral branches at positions 18–24. R, rooted parent node; R + 1, next distal non-rooted parent node and so on. Values at positions R6 to R10 were not significantly different and pooled. Data are means of eight replicated plants + s.e.
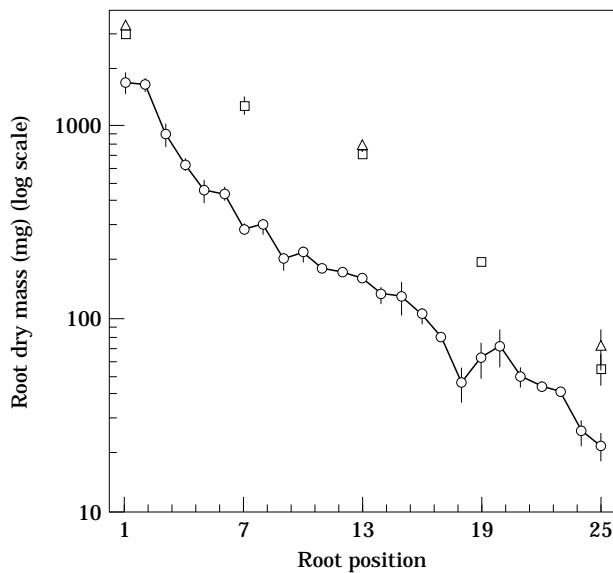

FIG. 5. Influence of rooting treatments on the dry mass of roots located along the parent axis. The parent axis was freely rooted (O), rooted at every sixth node (■), or rooted at every twelfth node (△). Roots are numbered in ascending order towards the apex. Data are means of eight replicates ±s.e.

TABLE 3. *Total shoot and root dry mass of* Trifolium repens *plants subjected to different rooting treatments. Data are means of eight replicates. Values with a common letter within rows do not differ significantly at* P = 0·05.

	Rooting treatments		
	FR	RR6	RR12
	Number of rooted nodes on parent axis		
	25	5	3
Shoot (g)	36·4[a]	29·7[b]	27·7[b]
Roots (g)	7·8[a]	5·2[b]	4·1[c]

DISCUSSION

Initiation of inflorescences and vegetative outgrowth

A decrease in the number of rooted nodes on the parent axis promoted the initiation of inflorescences (Table 1). Two factors appeared to be involved in this initiation: (*a*) since inflorescences occurred on nodes produced after the severance of the parent axis stolon and almost exclusively in plants with restricted rooting it is likely that the sudden change of the root/shoot ratio, due to the severance of the stolon, triggered the initial initiation of inflorescences; (*b*), the number of nodes with inflorescences increased with the increasing number of non-rooted nodes suggesting that the increasing distance of the apical bud relative to the nearest proximal rooted node promoted the initiation of inflorescences. It is generally accepted that, in clonal plants, limited

roots which were at the same nodal position on freely rooted plants (Fig. 5). However, this compensation was not enough to prevent a loss in the total dry mass of roots (Table 3). In contrast total dry mass of the shoot was less affected by the number of rooted nodes.

resources (photosynthates or mineral nutrients) reduce the probability of a ramet flowering (Davy, 1987). For *T. repens*, the relationship between availability of resources and initiation of inflorescences is not clear (Thomas, 1987*b*). In our experiment, limitation of resources was related to the selective partitioning of mineral nutrients among branches and the parent axis and not by the availability of nutrients at roots. Since growth of the parent axis was not affected by the rooting treatments, the increase in formation of inflorescences was controlled presumably by plant hormones rather than the result of nutrient stress within the apical bud. However, more information about the floral stimulus (Metzger, 1995) is needed to explain the relationship between rooting and initiation of inflorescences.

Initiation of axillary bud outgrowth was affected by the position of the bud relative to the rooted node. However, a clear distinction must be drawn between the probability of outgrowth of an axillary bud and the time when outgrowth occurs, since both factors influence the proportion of branched nodes per plant at a given time. At harvest, 6·5% of the axillary buds on the parent axis were classified as dormant in plants with restricted rooting as compared to 1·6% in the freely rooted plants. This weak response agrees with a report of weak correlations between rooting at a node and the probability of outgrowth of the associated axillary bud (Newton and Hay, 1994). It seems that roots stimulate outgrowth of axillary buds by plant hormones (e.g. root-produced cytokinins) rather than by providing mineral nutrients (Thomas, 1987*a*). But the development of axillary buds is more complex and probably controlled by the balance of several plant hormones (Tamas, 1995). Nevertheless, outgrowth was fast and occurred with little temporal variation only when the bud was located at the rooted node or two nodes distal to the root (Fig. 3). This indicates that where buds have direct vascular connections to a particular root (see Devadas and Beck, 1972; Thomas, 1987*c*) outgrowth is faster and more likely to be stimulated by the presence of that root.

Branch development

Branch development was dependent upon both the number of rooted nodes on the parent axis and the position of the parent node. The reduction in the number of rooted nodes resulted in compensatory growth of remaining roots (Fig. 5) and of branches emerging from rooted parent nodes (Fig. 4). Due to this compensatory growth, a reduction of 88% in the number of rooted nodes in treatment RR12 reduced the total root dry mass by 47% but the total shoot dry mass by only 24% (Table 3). Therefore compensatory growth introduces the possibility that there is a threshold for the number of rooted nodes per plant above which shoot biomass and thus harvest index is not affected by the number of rooted nodes. Factors associated with supply of assimilates and mineral nutrients may have contributed to the faster development of the roots and their associated branches in treatments with restricted rooting: (*a*) restriction in rooting increased the number of parent leaves per root and thus increased the carbohydrate supply per root. This may explain the higher dry mass of young roots (position

25, Fig. 5) in RR6 and RR12 as compared to FR. (*b*) When young nodes are in contact with a moist surface, the outgrowth of a root occurs before that of an axillary bud. This means that at the time axillary buds grew out of rooted parent nodes, the buds in restricted rooted plants were supplied by larger roots compared to freely rooted plants. These larger roots had a positive influence on branch development because young branches at rooted nodes were markedly heavier in plants with restricted rooting (Fig. 4). Finally (*c*), when the carbohydrate balance of such young branches becomes positive, considerable amounts of carbohydrates are translocated from the branch to the parent axis (Robin, Guerin and Guckert, 1989; Chapman, Robson and Snaydon, 1992*a*), providing up to 84% of the carbohydrate used for growth of the root at the parent node (Chapman, Robson and Snaydon, 1992*b*). Due to the faster branch development, roots on plants with restricted rooting were then supplied by better developed branches as compared to freely rooted plants and this in turn may have favoured root growth.

Development of branches was also influenced by the position of the branch in relation to the nearest rooted parent node. Branches emerging from node R+2 were superior to the other branches in all characteristics (Table 2, Figs 3 and 4). Analyses suggest that there are no direct cross-connections between vascular bundles along either side of stolons of *T. repens* (Devadas and Beck, 1972; Thomas, 1987*c*). In addition, a significant proportion of resources exported from a nodal root of *T. repens* is transported into the associated branch (Chapman and Hay, 1993; Kemball and Marshall, 1994) and, within the parent axis, distributed mainly acropetally (Nelson and Brady, 1953; Hoshino, 1974; Kemball and Marshall, 1994). For these reasons, resources exported from a root and distributed within the parent axis are more likely to move into distal branches located on the same side of the parent axis as the source root (e.g. branches at parent nodes R+2 and R+4) rather than into branches on the opposite side. The finding that the development of the branches emerging at parent nodes R+2 is superior to that of branches at R+1 is consistent with reports that the distribution pattern of recently assimilated radioactive phosphorus (^{32}P) showed limited allocation to branches at parent nodes R+1 (Hoshino, 1974; Hay and Sackville Hamilton, 1996; Lötscher and Hay, 1996*a*). However, there is genotypic variability in intra-plant distribution of mineral nutrients when plants grow with various rooting patterns, suggesting that genotypes of *T. repens* differ in resource partitioning and thus in their growth strategies (Lötscher and Hay, 1996*b*).

Branch establishment

Branch establishment is regarded as having occurred when the root system on a branch is capable of supplying water and mineral nutrients to sustain growth and development of the branch. Under field conditions where root formation on the parent axis is locally suppressed (e.g. missing contact with moist soil surface) branches have to spread to find microsites that trigger root outgrowth. In our experiment a

considerable proportion of branches on non-rooted parent nodes failed to produce any elongation of internodes (Table 1 and Fig. 4). Such axillary branches presumably had low probability of ever fully establishing.

Conclusions

The whole plant response to restricted root formation was to maintain continuous growth of the parent axis and to invest into compensatory growth of the branch at the rooted node. The strong development of the branches originating from the rooted node and the second node distal to a rooted node indicates that at least two branches per rooted node have a high probability of exploring the sward. Consequently, optimal branching of a plant can be expected when about 50% of the nodes, regularly distributed along the parent axis, are rooted. Growth was slow for axillary buds whose development depended on the basipetal movement or cross-transport of mineral resources and possibly of plant growth regulators within the stolons. The high variability in the development of branches caused by a single factor (root formation) identifies another complex topic for consideration in the development of plant growth models. Furthermore, this study indicates that careful observation of the rooting pattern of *T. repens* plants is needed when the influence of other factors, for example light quality or defoliation, on branching are investigated.

ACKNOWLEDGEMENTS

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