ROOT ARCHITECTURAL AND MORPHOLOGICAL RESPONSE OF *PINUS NIGRA* ARN. AND *QUERCUS ROBUR* L. TO NUTRIENT SUPPLY AND ROOT DENSITY IN THE SOIL

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ABSTRACT - This study used seedlings of *Quercus robur* L. and *Pinus nigra* Arn. to investigate the response of root systems to nutrient supply and root density in the soil. Variation in nutrient supply was tested by using two different concentrations of nitrate and phosphate. The influence of root density was tested by spacing seedlings to provide a density of 100, 67 and 25 plants m^{-2}. At the end of the treatments the two species presented two different root topologies which apparently each derive from a proper ontogenic specificity. Nevertheless, the data clearly indicate that competition for nutrients and root density have affected both root system topologies by inducing a considerable modification in root traits such as total root length and number of apices. These modifications were different when measured in first-or second-order root laterals. This study reveals that the response of these two species to heterogeneity in soil resource distribution and root competition is regulated by root turnover. A better understanding of the response of roots to environmental signals improves our knowledge of the interaction between plants and the soil ecosystem in forests.

KEY WORDS - Root systems, soil fertilization, root density, *Quercus robur* L., *Pinus nigra* Arn.

INTRODUCTION

Temporal and spatial heterogeneity in soil resource distribution is relatively great in forests (Fitter, 1994; Farley & Fitter, 1999; Lechowicz & Bell, 1991), but ecological theories concerning the relationship between plant traits and resource supply have focused primarily on leaf structure and leaf physiology (Reich *et al.*, 1997), neglecting other organs such as roots. The limited information that exists for roots suggests the occurrence of plasticity in tree root system structure and root morphology in response to environmental variability (Fitter, 1994; Nilsen & Orcutt, 1996; Fitter, 1999; Jackson *et al.*, 1999; Fitter & Hay, 2002; Lenschner *et al.*, 2004). Furthermore, there are studies that have investigated root system development in response to varying soil resources, location and phenology, although mainly non-woody species have been investigated. As a consequence our understanding of the interaction between root systems and the remainder of the soil ecosystem in forest habitats remains very patchy (Fitter, 1991).

The response of both root system architecture (RSA) and size to the soil environment, and especially to the availability and distribution of nutrients, is highly variable (Itoh & Barber, 1983; Robinson, 1994; Thaler & Pagés, 1998; Zhang & Forde, 1998; Williamson *et al.*, 2001; Linkor *et al.*, 2002). In nutrient-limiting conditions root systems generally have a very different aspect from those of root systems in nutrient-rich environments, but not all plant species respond in the same fashion to heterogeneity in soil nutrient supply (Farley & Fitter, 1999) and not all nutrient ions elicit response (Drew, 1975). This variability is probably due to the exceptionally plastic development of the root system, in comparison with other plant organ systems (Fitter *et al.*, 1999) Furthermore, plasticity itself may derive from the development of the root system via the continual propagation of newly formed meristems which may undergo differential regulation by environmental signals (Williamson *et al.*, 2001).

Attempts to reveal the developmental mechanisms that lead a plant to modify its root system in response to soil nutrients have mainly been conducted for the model plant *Arabidopsis thaliana*. The almost exclusive use of this plant in studies of root system development is in part due to the fact that its genome has been now completely described, enabling future use of molecular tools to investigate the chain of events underlying the plant response to environmental signals. It is already known that nitrate supply suppresses lateral root development (Zhang *et al.*, 1999), acting mainly on the rate of lateral root elongation rather than on lateral root initiation (Zhang & Forde, 1998). Nitrate diffusion in soil is three to four orders of magnitude faster than that of phosphate and this high mobility means that roots 1 cm apart may compete for nitrate after as little as 24h (Tinker & Nye, 2000). Given the high mobility of nitrate a low root density should be sufficient to capture all the nitrate in a given volume of soil (Hodge *et al.*, 1999), raising the question as to why root proliferation occurs (Robinson, 1996) [N.B. Fitter (1999) answers Robinson's question 'Why do plants bother?': root proliferation is advantageous only when there is competition between roots and resources must be acquired as rapidly as possible-proliferation is an important competitive advantage mainly in productive habitats, for the acquisition of all nutrients]. In contrast, phosphate has shown to be often the limiting nutrient for plant growth because of its low mobility in soil. Therefore, it is not surprising that phosphate can also have a profound effect on RSA (Williamson *et al.*, 2001). A primary adaptation to low P availability appears to involve postembryonic developmental changes in the root system, which are directed towards enhancing P uptake. These include altered branching patterns, total root length, root hair elongation, and lateral root formation (Dinkelaker *et al.*, 1995; Bates & Lynch, 1996; Borch *et al.*, 1999; Williamson *et al.*, 2001). Root hairs and lateral roots assist the acquisition of P by exploring a greater soil volume and by increasing the absorptive surface of the root (Lòpez-Bucio *et al.*, 2002).

While awaiting the description of the genome of a true woody species it is of some interest to accumulate information at a morphological level similar to those collected for *Arabidopsis thaliana*, regarding the response to nutrient supply of the root system in species used in forestry. However, an obstacle to these investigations is the labour-intensive necessity of measuring the morphological traits of large root systems. To this end, we have recently introduced in our work on roots computerised systems based on image analysis (Chiatante *et al.*, 2002) that enables rapid measurement of root traits (length, diameter, number of root apices, root volume, root surface) even in complex root system of species used in forestry research. These methods are reliable, reduce the possibility of affecting data by artefacts, and enable the storage of a large amount of data which may be successively analysed for statistical purposes. In this paper, we have applied the computerised image analysis of root systems to investigate the effect of different nutrient concentrations and root densities for seedlings of two woody species (*Quercus robur* L. and *Pinus nigra* Arn.). These species were selected as they represent angiosperm and gymnosperm species (respectively) frequently used in forestry. As suggested by Koike *et al.* (2003) the effect of nutrient supply on root growth was investigated by manipulating nitrate and phosphate concentration using a stationary bulk fertilization approach (Timmer & Munson, 1991; Miller & Timmer, 1994). For the root density experiment, we tested three different seedling spacing distances. The effects of treatment combinations on the root systems of *Q. robur* and *P. nigra* are discussed.

MATERIALS AND METHODS

Preparation of plant material

The nursery site of the Viterbo University, used to produce seedlings, is situated 325 m a.s.l. (42°41'N, 12°10'W). Annual rainfall at the nursery is 954 to 1166 mm yr^{-1} and 103-to 163 mm during summer months. The cold season occurs between October and May, when the mean minimum temperature is -0.3°C.

One-year seedlings of *Pinus nigra* produced in the nursery were transplanted in the autumn of 2001 to a transplant bed, at three different spacing densities (10 x 10 cm=100 plants m^{-2}; 10 x 15 cm=67 plants m^{-2} and 20 x 20 cm=25 plants m^{-2}) and three levels of fertilization, with N supplied as ammonium nitrate (NH_4NO_3) and phosphorus supplied as phosphate (P_2O_5) (1. N 0 g m^{-2} and P 0 g m^{-2}; 2. N 30 g m^{-2} and P 57 g m^{-2}; 3. N 45 g m^{-2} and P 85.2 g m^{-2}). Acorns collected from a natural stand of Quercus robur near Rome (Castel di Guido), in November 2001, were sown directly in the nursery and subjected to the same density and fertilization treatments. In order to avoid the drawbacks of possible adverse weather conditions, the beds were covered with transparent plastic tunnels within which an irrigation system was set up. Thus a high germination percentage was obtained for all treatments. Aliquots of seedlings of both species were collected for measuring root traits during the early spring 2003.

Root trait measurement and statistical analysis

After the period of growth in the nursery beds where different concentration of soil fertilization and spacing density had been applied, the seedlings were excavated with care by a spade and were freed from the soil by washing them in running tap-water. The root system of each seedling was placed on the scanner taking care of orienting each lateral roots as close as possible to its natural orientation observed during excavation. Images of excavated root systems were scanned with a scanner calibrated with the WhinRhizo software package, 3.10 version (Regent Instruments Inc., Quebec, Canada). Computerised image analysis provided morphometric root measurements such as root length and number of root apices. Calibration of WhinRhizo software with diameter intervals enabled to distinguish between roots belonging to a specific root order. By this method it was possible to subdivide the root system in root categories and root traits were measured for each category. Data of each sampling of five replicates were analysed separately for each species using a General Linear Model (GLM) with fertilization and spacing density as fixed factors. If necessary, data were transformed to meet the requirements of parametric analysis. Differences between means were tested using a LSD test with a level of significance of $P<0.05$.

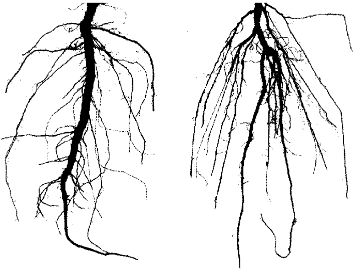

FIGURE 1 - The root systems of *Quercus robur* (left) and *Pinus nigra* (right) seedlings following excavation.

RESULTS

The aspect of the excavated root systems of *Q. robur* and *P. nigra* seedlings, respectively, before scanning is shown in FIGURE 1. Image analysis of this, and other, images reveals that a considerable difference in RSA developed during growth. In fact, the first order lateral roots of *P. nigra* seedlings showed a greater diameter and a conical shape, tapering distally. In contrast, first order lateral roots of *Q. robur* seedlings have a less conical and more cylindrical shape. The second order lateral roots of both species did not show morphological differences.

Statistical analysis determined that the distribution of the parameters examined was strongly bimodal. Based on this, the species factor has not been taken into account in the factorial design and each species was treated separately (TABLE 1). The first morphological parameters measured were shoot height and rooting depth at the end of treatments. In this case, the distance from the root collar to the more distal root tip indicated the rooting depth whereas the distance from root collar to the highest stem apex indicated the seedling height. The results of these measurements are shown in FIGURE 2 and reveal that rooting depth of *Q. robur* was unaffected by root density in the absence of any nutrient addition to the soil. When both low and high fertilization treatments are considered, rooting depth varied in response to seedling density; however, the maximum value observed remained the same one found as in absence of any soil fertilization. Shoot height was constrained by root density for all treatments. In the case of *P. nigra* seedlings, shoot height was unaffected by the degree of fertilization and root density, whereas considerable variability was observed in root depth. This appeared to be influenced by root density, especially when the degree of fertilization was higher. However, in many cases the differences measured were not significant (FIGURE 2).

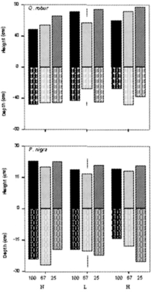

FIGURE 2 - Stem height and rooting depth of *Quercus robur* and *Pinus nigra* at three fertilisation levels and spacing densities. N represents the control where no fertilization is present. L and H represent respectively the lower and higher values of nitrate and phosphate concentrations used as fertilization supplies. 100, 67 and 25 are the number of seedlings present per m^{-2}. The ordinate axis presents the value of shoot height and root depth. Values are means of five replicates + 1SD.

Higher root density decreased total root length (TRL) for *Q. robur* in the absence of fertilization (FIGURE 3 A), but not at the highest degree of fertilization. TRL remained nearly constant at the lowest level of fertilization. The highest value of whole TRL was found in seedlings that had been treated with low fertilization and a medium value of root density (FIGURE 3 A). The length of first order lateral roots followed the same pattern shown by TRL (FIGURE 3 C), whereas some difference was found in the length of second order lateral roots (FIGURE 3 E). In this case, the TRL value was lower when the degree of fertilization was increased at the lowest value of root density, but was highest when soil fertilization and root density were highest. Even in the case of second order laterals the highest value of TRL was found in seedlings treated with low fertilization and a medium value of root density.

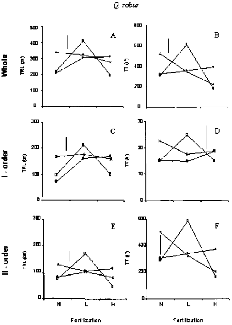

FIGURE 3 - Total root length (TRL) and total root tips (TT) of Quercus robur at different fertilization concentrations and spacing densities. N represents the control where no fertilization is present. L and H represent respectively the lower and higher values of nitrate and phosphate concentrations used as fertilization supplies. For spacing treatments: filled circle (Σ)=100 seedlings m^{-2}; empty square (□)=67 seedlings m^{-2}; cross (×)=25 seedlings m^{-2}. Panels A and B present the values referred to the whole root systems. Panels C and D present the value referred to the first order root laterals; Panels E and F present the value referred to the second order root laterals. Values presented are means of five replicates analysed by one-way ANOVA. The bars indicate the Least Significant Difference (LSD, $P<0.05$) used for pair wise comparisons.

When we measured the total number of root tips (TT) in the whole root system, or separately between first and second order lateral roots, we found similar variability to that described for the TRL. The TT value decreased when the soil was fertilized at lower root density, but increased when the soil was fertilized at high root density. Also in this case the highest value was observed for seedlings treated with low soil fertilization at a medium value of root density. This response of root tip number to the treatments was investigated also for both first and second order laterals. *Quercus robur* produced a total of 300 cm of root material from almost 500 root tips during our experiments (FIGURE 3 A, B), approximately half of which was produced by a minority (4%) of first order lateral roots.

When the effects of fertilization and root density upon TRL or TT were measured for *P. nigra*, we found a greater variability than for *Q. robur* although differences were almost not significant (FIGURE 4 and TABLE 1). In particular, when the length of first order lateral roots was examined, a significant difference was found only at the lowest root density, which showed the highest value when the soil remained unfertilized (FIGURE 4 C). Furthermore, significant differences in second order lateral roots were found for root length with greater soil fertility, particularly at high root densities when the lowest value was achieved (FIGURE 4 E). Similar results were obtained for the number of root tips (FIGURE 4 D). Less significant differences were observed for the number of root tips when the degree of fertilization was increased (FIGURE 4 D). A significant lower number of root tips was found for second order laterals grown at the highest soil fertility in presence of a high root density (FIGURE 4 F). However, for *P. nigra* we observed that first order lateral roots representing only 6.5% of the total number of roots found in a root system accounted for nearly 50% of the total root length measured (FIGURE 4 D).

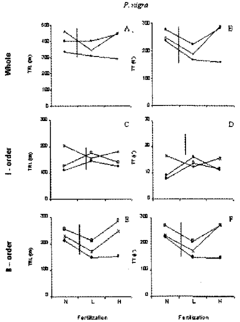

FIGURE 4 - Total root length (TRL) and total root tips (TT) of Pinus nigra at different fertilization concentrations and spacing densities. N represents the control where no fertilization is present. L and H

represent respectively the lower and higher values of nitrate and phosphate concentrations used as fertilization supplies. In regard of spacing treatments: filled circle (·)= 100 seedlings m^{-2}; empty square (□)= 67 seedlings m^{-2}; cross (×)= 25 seedlings m^{-2}. Panels A and B present the values referred to the whole root systems. Panels C and D present the value referred to the first order root laterals; Panels E and F present the value referred to the second order root laterals. Values presented are means of five replicates analysed by one-way ANOVA. The bars indicate the Least Significant Difference (LSD, $P<0.05$) used for pair wise comparisons.

DISCUSSION

Differences in topology influence both exploration and transport characteristics of the root system, impacting on the ecological tolerance of individuals and hence the field distribution of species (Bell & Sultan, 1999). The two extreme types of root system topology reported in literature are the "herringbone" topology (a single main root with one order of laterals) and the "dichotomous" topology (all external links form new branches with equal probability) (Fitter, 1987) although the occurrence of an intermediate topology has not been excluded. Fitter (1991) and Fitter & Stickland (1991) indicated that herringbone-like topology is highly effective in terms of exploitation and exploration and may be preferable in nutrient-poor conditions (for mobile nutrients such as nitrate) with shorter link length occurring in response to higher nutrient availability. The dichotomous topology is most effective at exploiting a restricted soil volume, particularly if transport within the soil limits uptake, but it would be of limited effectiveness in exploring spatially heterogeneous soil (Fitter, 1991). The intermediate topology would be functionally intermediate too (Fitter, 1991). Fitter (1987) and Fitter *et al.* (1991) predicted that root systems with herringbone pattern exploit nutrients more efficiently, but are also associated with higher construction costs than root systems with dichotomous branching pattern.

At the end of the growth period, the root system of the two species examined in this study present a very different root organization due to the deployment of two completely different topologies. This confirms that root topology and morphology varies widely between species, between individuals of a species and even within an individual root system (Fitter, 1991). The root system of *Q. robur* may certainly be classified as a herringbone-like topology in which a large taproot determines a central structure from which first order lateral roots depart in different directions. All the lateral roots maintain almost the same diameter along their length but diameters differ according to the order of root to which they belong. In the case of *P. nigra*, the root system may be more easily associated with a "dichotomous" topology. In fact, the first order lateral roots have different diameters and all follow a conical shape, tapering distally. However, we suggest that the deployment of different typologies remains to be ascribed primarily to an intrinsic existing "ontogenic plasticity" (Gedroc *et al.*, 1996; Pigliucci *et al.*, 1996) whereas the treatments influence morphological aspects referring to a specific root topology.

Regarding the morphological responses to treatments, it is first necessary to consider that optimality theory would predict that trees optimise root operation by maximising nutrient and water uptake per plant with a minimum resource investment into the root system. Furthermore, it has been suggested that in habitats with low nutrient and/or water supply, trees could respond with alternative strategies, e.g., with elevated specific uptake rates, a lower fine root turnover rate, a smaller fine root system size, or alternatively with a larger fine root system size if uptake would increase over-proportionally by additional roots (Lenschner *et al.*, 2004). In our experiments, it must be taken into account that the spacing treatments are responsible for generating a different level of root density in the below-ground compartment. This fact greatly influences much the response of the root system to environmental signals by introducing an additional factor represented by the density of roots in a certain volume of soil. However, in the case of the whole root system the value of TRL in *Q. robur* appears to be negatively influenced by the root density when soil remains unfertilized, but is positively influenced when soil fertilization increases. This suggests that for *Q. robur* competition for nutrients may determine a decrease in the number of new roots produced probably as an effect of root turnover. The root system recovers its growth in more fertile soil as shown by the increase of the degree of ramification (increase in the number of first and second order lateral roots measured as number of root tips). This effect is particularly evident with a medium degree of fertilization. The fact that the number of first order laterals doubled only when the soil was fertilized to a small extent and root density was low, confirms the negative effect on root turnover of high competition for nutrients. The fact that in absence of soil fertilization and the presence of low root densities, we observed the highest value of root length (independently from the root category considered) also explains why under these conditions the shoots are larger; more biomass could have been invested in the construction of the above ground portion of the plant as consequence of an increased availability of nutrients acquired by a larger root system. Our data are consistent with observations of other authors, regarding root growth in nutrient-poor soils (Reynolds & D'Antonio, 1996).

The response of *P. nigra* root systems to the treatments is more difficult to interpret, with high variability in root length and the number of root tips. The only clear indication is that high root density suppresses the length and number of first order laterals in the absence of soil fertilization, whereas the development of second order laterals is suppressed in highly fertilized soils. Therefore *P. nigra* seedlings respond to low nutrient availability by increasing the number and length of first order lateral roots which results in a more branched root system. At the same time, in this species branching seems to be inhibited by competitive restriction of rooting volume as indicated by the lowest value of root tips belonging to the second order root category.

With regard to the observed changes in root morphology, our results support the hypothesis that a shoot-derived signal suppresses lateral root growth when nitrate is abundant, but a lack of nitrate perceived at the lateral root tip stimulates root elongation (Zhang & Forde, 1998). The formation of a highly branched root system, in response to limited nutrient availability, has been interpreted as a consequence of the allocation of carbon and energy resources to produce a root system capable of exploring large areas of the upper soil layer, where nutrient-rich patches are normally present (Stitt & Rudiger-Scheible, 1988). The effect of this response is that plants grown with plenty of nutrients often have smaller root systems (usually expressed as dry weight) as a fraction of the size of the shoot than do plants grown in nutrient-deficient conditions, especially when the nutrients in question are N, P or K. However, this response appears to be independent of the plant's demand and the severity of the nutrient shortage (Robinson, 1996). Regarding phosphate, the literature reports that higher phosphate availability increases the total number of root branches and the total root system length but decreases lateral root density (Williamson *et al.*, 2001). Furthermore, it seems that lower phosphate favours lateral root growth over primary root growth by reducing primary root elongation, increasing lateral root elongation, and increasing lateral root density (Williamson *et al.*, 2001). In our experiments there is no possibility to distinguish between the separate effect of nitrate and phosphate supply. However, it seems clear that the lack of fertilization induces the formation of new meristems that are in turn responsible for the formation of new laterals. This event occurs only in the absence of competition (high root density), with suppression of root growth at high densities overcome only by increasing either nutrient availability or the spacing of individual trees.

To conclude, the present study demonstrated that *Q. robur* and *P. nigra* seedlings are characterized by different root topology and responses to soil nutrient availability and planting density. These different responses involve variations on both the length and the level of ramification of the root system. The physiological relevance of these differences for nutrient acquisition demand further attention. The importance of studies such as the one reported here lies in improving our knowledge of fine root dynamics in relation to environmental signals in tree species and this is necessary to quantify energy and nutrient flows in forest ecosystems (Persson, 1983).

RIASSUNTO

Questo lavoro studia la possibile risposta degli apparati radicali di piantine di *Quercus robur* and *Pinus nigra* rispetto a variazioni della distribuzione di nutrienti e della densità delle radici nel suolo. Nel caso di variazioni di nutrienti questo esperimento si è avvalso di diverse concimazioni del suolo a base di nitrati e fosfati. Per lo studio della densità delle radici sono state usate tre diverse concentrazioni di piantine m^{-2} (100-67-25). Alla fine dei trattamenti l'escavazione degli apparati radicali ha messo in evidenza lo sviluppo di due differenti tipologie di apparati radicali. Queste due tipologie sembrano essere il frutto di una diversità ontogenica e non dei trattamenti imposti. Comunque i dati mettono in evidenza che, nell'ambito di una autonomia di risposta, i trattamenti hanno comunque influenzato in entrambe le specie parametri morfologici delle radici come la lunghezza totale delle radici o il numero di apici radicali presenti in un apparato radicale. Queste risposte morfologiche sono state diverse se misurate su radici di primo o secondo ordine; peraltro, tutte potrebbero essere fatte risalire a differenze della regolazione del ciclo vitale delle radici. L'importanza di questi risultati è legata al fatto che essi incrementano le conoscenze della risposta dell'apparato radicale a stimoli ambientali consentendoci di comprendere meglio l'interazione esistente tra pianta ed ecosistema suolo.

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TABLE 1 - F and P values of ANOVA (GLM) for the morphological plant traits of *Q. robur* and *P. nigra* with the factors fertilization and spacing as fixed factors. Non-significant interactions were excluded from the model (-). Boldface *P* values are significant at a probability level of $P<0.05$.

		Quercus robur				*Pinus nigra*		
		FERTILIZ	DENSITY	F x D		FERTILIZ	DENSITY	F x D
Parameter	Value	(df = 2)	(df = 2)	(df = 4)		(df = 2)	(df = 2)	(df = 4)
TRL whole root system	F	7.902	1.531	5.469		0.484	2.899	-
	P	***0.001***	*0.230*	***0.002***		*0.620*	*0.067*	-
TRL 1th order	F	10.834	3.065	4.846		0.127	2.414	-
	P	***<0.001***	*0.059*	***0.003***		*0.881*	*0.102*	-
TRL 2nd order	F	8.062	0.209	8.807		1.929	3.241	-
	P	***0.001***	*0.813*	***<0.001***		*0.159*	***0.049***	-
TT 1th order	F	0.240	0.856	1.983		0.902	1.480	-
	P	*0.788*	*0.433*	*0.118*		*0.414*	*0.240*	-
TT 2nd order	F	8.841	0.022	9.006		1.788	2.190	-
	P	***0.001***	*0.979*	***<0.001***		*0.180*	*0.125*	-
Height	F	6.499	6.208	-		3.514	3.464	-
	P	***0.003***	***0.003***	-		***0.035***	***0.037***	-