Gorse is a 'facultative' N_2 fixer

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Abstract

Many legumes reduce their atmospheric N_2 fixation per unit biomass in response to increased soil N availability but there are reports that some maintain a constant rate of N_2 fixation per unit biomass regardless of soil N levels. These different responses to soil N availability have been described, respectively, as 'facultative' and 'obligate' N_2 fixation strategies. Views in the literature differ if gorse is a facultative or obligate N_2 fixer. Here, firstly, the proportion of N derived from the atmosphere (%Ndfa) was assessed for mature gorse plants mainly in hedges bordering intensive agricultural land at different sites in the Selwyn district, Canterbury, New Zealand using the ^{15}N natural abundance technique. Secondly, the effect of nitrate (NO_3^-) supply on %Ndfa was determined for gorse seedlings under glasshouse conditions using $^{15}NO_3^-$. Under field conditions, values ranged from 14.7-88.0 %Ndfa. In the glasshouse, %Ndfa values decreased from 97 when no N was supplied to 24 %Ndfa when N supply was increased to the equivalent of 200 kg N/ha. It is concluded that gorse shows a facultative N_2 fixation strategy.

Keywords: legume, nitrate, ^{15}N natural abundance, nitrate reductase activity, gorse, *Ulex europaeus*

Introduction

Generally, legume (Fabaceae) species can fix atmospheric nitrogen (N_2) via symbiotic bacteria ('rhizobia') in root nodules and also utilise soil inorganic N (nitrate (NO_3^-) and ammonium (NH_4^+)) when available (Andrews *et al.* 2013). There are many reports for legumes of increased reliance on soil N in comparison with N_2 fixation as soil N levels increase (Andrews *et al.* 2011; Barron *et al.* 2011). The ability to adjust N_2 fixation per unit biomass in response to different soil N availability has been termed a 'facultative' N_2 fixation strategy (Menge *et al.* 2009). However, there are reports that some legumes, for example, common broom (*Cytisus scoparius*), common vetch (*Vicia sativa*) and *Vicia americana* maintain a constant rate of N_2 fixation per unit of biomass regardless of soil N availability; this has been termed an 'obligate' N_2 fixation strategy (Menge *et al.* 2009, 2015; Drake 2011).

Gorse (*Ulex europaeus*) is a perennial woody legume shrub native to Western Europe which is a common hedge around pastures and other agricultural land on the Canterbury Plains in New Zealand. However, it is also a serious invasive weed in mature pastures, as well as riparian areas, marginal land and forest margins (Popay *et al.* 2010; Magesan *et al.* 2012; Delerue *et al*. 2014). Magesan *et al.* (2012) reviewed the literature on N cycling in gorse-dominated ecosystems in New Zealand. It was argued on the basis of available data that as soil inorganic N increased, gorse N_2 fixation decreased. For example, Thornton *et al.* (1995) using ^{15}N labelled NO_3^- and NH_4^+ reported that for 2 year old plants, N_2 fixation was the major source of N at 0.25 mol/m^3 applied NH_4^+ or NO_3^- but that soil N was the major source of N at 5 mol/m^3 NH_4^+ or NO_3^-. Growth (total plant dry weight) was similar at low and high N supply but nodule dry weight and N_2 fixation were an order of magnitude lower at high N. In contrast, Drake (2011) reported that a comparison of $\delta^{15}N$ natural abundance of shoots and in NO_3^--N of surface and ground water indicated that 69.5-88.4 % of N was derived from the atmosphere (%Ndfa) for gorse growing in riparian areas of 'N-saturated' intensive agricultural land in Canterbury, New Zealand. This finding, along with the assumption that data obtained from an N balance study of common broom (*Cytisus scoparius*) in a glasshouse also applied to gorse, led Drake (2011) to conclude that gorse is likely to be an obligate N_2 fixer. Gorse has colonised approximately one million ha of land in New Zealand (Magesan *et al*. 2012) thus if it is an obligate N_2 fixer, then this could result in an important continuous source of N and ultimately NO_3^- pollution into rivers and lakes. However, in relation to Drake (2011), N levels in riparian areas are likely to be much lower than in the adjacent agricultural land and the study on broom added 0.1 g NO_3^--N/m^2 each week over 9 months with harvests at 2, 4, 7 and 9 months after planting. This is the equivalent of 1 kg N/ha added each week which is a low input relative to the N requirements of dairy pastures or cereal crops over the growing season (Andrews *et al.* 2007; Cameron *et al.* 2013). Thus, the conclusion of Drake (2011) that gorse is an obligate N_2 fixer may not be valid.

This study assessed if gorse is a facultative or obligate N_2-fixer over the range of NO_3^- levels likely to occur in intensive agricultural soils. Firstly, %Ndfa was assessed for mature gorse plants mainly in hedges bordering intensive agricultural land at different sites in the Selwyn district, Canterbury, New Zealand using the ^{15}N natural abundance technique. Secondly,

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the effect of NO_3^- supply (0-200 kg N/ha) on %Ndfa was determined for gorse seedlings under glasshouse conditions using $^{15}NO_3^-$.

Materials and methods

Field study

The %Ndfa by mature gorse bordering agricultural land in the Selwyn district was assessed using the ^{15}N natural abundance technique (Unkovich *et al.* 2008; Andrews *et al.* 2011). Six 11-22 km 'transects' were run outwards along roads from Lincoln township, Selwyn, Canterbury (43º39´S 172º29´E) during October 2013. At three points along each of the six transects (~ 1 km, 5-11 km and 11-22 km), ~ 30 cm shoot samples of gorse and two or three reference plant species within 3 m of the gorse were taken. Woody as opposed to herbaceous plants were used as reference as they have a similar duration of growth to gorse and it was thought that they were more likely to access the same soil N pools as gorse (Unkovich *et al*. 2008). The reference plants from each sampling site were pooled for analysis. Depending on sampling point, the reference plants were in the genera *Cedrus, Chamaecyparis, Crataegus, Cupressus, Eucalyptus, Fraxinus, Olearia, Pinus, Pittosporum, Populus, Prunus, Quercus, Rosa, Rubus, Salix, Sambucus* and *Thuja.* The material was ground and analysed for $^{14}N/^{15}N$ with a Sercon (Crewe UK) GSL (gas, liquid, solid) elemental analyser attached to a Sercon 20-22 isotope ratio mass spectrometer.

The %Ndfa for gorse at each sampling point was determined as:

$$\% \text{ Ndfa gorse} = \frac{\delta^{15}N \text{ reference plant} - \delta^{15}N \text{ gorse} \times 100}{\delta^{15}N \text{ reference plant} - B}$$

where 'B' is the $\delta^{15}N$ of gorse shoots fully dependent on N_2 fixation. The 'B' value was determined as -0.46 ± 0.10 from plants inoculated with *Bradyrhizobium* strain ICMP 19839 (International Collection of Microorganisms from Plants, Landcare Research, Auckland, New Zealand) grown in a glasshouse on N-free sand culture (Tan *et al.* 2012). Strain ICMP 19839 was isolated from gorse nodules and is an effective strain on this plant species.

Glasshouse experiments

For the three experiments, seeds of gorse were obtained from the Margot Forde Germplasm Centre, Palmerston North, New Zealand. Firstly, seeds were in sequence soaked in concentrated sulphuric acid for 30 minutes, rinsed with sterile water and soaked in hot (~ 60 °C) sterile water which was left at room temperature overnight. They were then transferred to 1.5 l pots (4 seeds/pot) containing 600 g of autoclaved N-free potting mix watered (sterilised reverse osmosis water) to field capacity. The potting mix base was 80% composted bark and 20% pumice (1-4 mm) to which was added 1 g/l agricultural lime (primarily calcium carbonate), 0.3 g/l superphosphate (9P-11S-20Ca; Ravensdown, New Zealand), and 0.3 g/l Osmocote (6 months, ON-OP-37K), 0.3 g/l Micromax trace elements and 1 g/l Hydraflo, all three obtained from Everris International, Geldermalsen, The Netherlands. The pH of the medium was 5.8. Plants were thinned out to two/pot, 2 weeks after sowing and pots were watered by weight to field capacity every 3 days. Experiment 1 was carried out over 12 weeks during October-December 2012. Experiments 2 and 3 were carried out side by side (starting dates four days apart), over 13 weeks, during March to June 2015. Day length was extended to 16 h with high pressure sodium lamps, if required.

Experiment 1 examined the effects of four different rates of N application (0, 5, 10 and 20 g N/m^2, 0-200 kg N/ha equivalant) supplied as KNO_3 on shoot and root dry weight (DW) and nitrate reductase activity (NRA), total plant N content and root nitrogenase (acetylene reduction activity, ARA) of rhizobial inoculated gorse. All plants were inoculated with 10 ml of *Bradyrhizobium* strain ICMP 19839 grown in yeast mannitol broth (YMB) (Vincent 1970) added to each pot in the first, second and third weeks after planting. Initially, there were ten replicates of each treatment (one replicate = one pot containing two plants). At harvest, plants from four replicates of all treatments were divided into shoot and root, dried at 60ºC for 7 days then reweighed. Shoot and root material was then pooled to give four replicates of total plant material for each treatment. This material was ground and total N content of 0.2 g samples was determined using a CN elemental analyser (Elementar VarioMax CN Elemental Analyser, GmbH, Hanau, Germany). *In vivo* NRA was determined in fresh shoot and root tissue of three replicates of plants of all treatments as described in Bungard *et al.* (1999). The remaining three replicates of all treatments were tested for nitrogenase activity via ARA (Cummings *et al.* 2009).

In Experiments 2 and 3, gorse and wheat (*Triticum aestivum,* cv. Spring Batten) were supplied the same N treatments as in Experiment 1 except that the KNO_3 was labelled at 10 atom% ^{15}N. There were four replicates of each treatment in Experiments 2 and 3, with one replicate equal to one pot containing two plants for gorse and three plants for wheat. In both experiments, shoot DW was determined on all plants from the four replicates of each treatment. Wheat shoots were sampled twice, approximately half way through and at the end of the experiments. The first cut was taken approximately 2 cm above the substrate and the second cut at substrate level. Dried material was ground and analysed for $^{15}N/^{14}N$ with a Sercon (Crewe, UK) GSL elemental analyser as for the field study.

The %Ndfa was determined via the ^{15}N isotope dilution method (Unkovich *et al*. 2008) as

$$\% \text{ Ndfa} = (1 - \frac{\text{atom\%}^{15}\text{N excess } N_2 \text{ gorse}}{\text{atom\%}^{15}\text{N excess reference plant}}) \times 100$$

Experimental design and data analysis

All experiments were of completely randomised design. An analysis of variance was carried out on all data (Minitab Version 16) with N rate as the fixed factor in all experiments and Experiment as a fixed factor across Experiments 2 and 3. Values obtained for Experiments 2 and 3 were not significantly different and were pooled for presentation. All effects discussed have an F ratio with a probability $P<0.01$. Variability quoted in the text is the standard error of the mean.

Results and Discussion

Views in the literature differ as to whether gorse is a facultative or obligate N_2 fixer and this difference could be an important factor determining N inputs into and N losses from gorse containing ecosystems in New Zealand (Thornton *et al.* 1995; Drake 2011; Magesan *et al*. 2012). It was assessed if gorse is a facultative or obligate N_2 fixer over the range of NO_3^- levels likely to occur in intensive agricultural soils. Firstly, %Ndfa was assessed for mature gorse plants mainly in hedges bordering intensive agricultural land at different sites in the Selwyn District, Canterbury, New Zealand using the ^{15}N natural abundance technique. At all sampling points (18 replicates), % N was greater and $\delta^{15}N$ was lower for gorse than for the reference plants. This suggests that gorse was fixing N_2 at all sites. The %N was 2.80 ± 0.06 and 1.79 ± 0.11 for gorse and reference plants, respectively. Values for %Ndfa for gorse ranged from 14.7-88% with an average of 48.7 ± 8.06% Ndfa. These values indicate that, over the area sampled, gorse obtains variable amounts of N from N_2 fixation and soil N assimilation and relies more on soil N when growing as a hedge in agricultural land than in riparian habitats adjacent to agricultural land (Drake 2011). This could be related to greater soil N levels in the immediate boundaries of agricultural land in comparison with riparian areas adjacent to agricultural land, but this was not tested.

In Experiment 1, total plant DW and N content were similar in treatments receiving 0 to 20 g N/m^2 (Table 1). This is in agreement with the findings of Thornton *et al.* (1995) that inoculated plants supplied 0.5 or 5.0 mol/m^3 N as NO_3^- or NH_4^+ showed similar growth. Values for ARA in Experiment 1 decreased consistently from 3.02 ± 1.33 to 0.09 ± 0.02 mmol ethylene/plant/h with increased N rate from zero to 20 g NO_3^--N/m^2. This indicates that N_2 fixation per plant decreased consistently with increased NO_3^- supply. In contrast, root NRA in particular, but also shoot NRA, increased with increased NO_3^- supply from 0 to 20 g NO_3^--N/m^2.

Table 1 Effect of different rates of applied nitrogen (N) as nitrate (NO_3^-) on total plant dry weight (DW) and tissue N content, root acetylene reduction activity (ARA) and root and shoot nitrate reductase activity (NRA) of gorse. Experiment 1. Variability shown is standard error of the mean. n=3-4. fw = fresh weight.

	Applied N (g N/m^2)			
	0	5	10	20
DW (g)	1.43 ± 0.23	1.34 ± 0.14	1.55 ± 0.26	1.24 ± 0.59
Tissue N (% DW)	1.85 ± 0.11	1.97 ± 0.25	2.03 ± 0.03	2.00 ± 0.17
ARA (μmol ethylene plant/h)	3.02 ± 1.33	1.59 ± 0.63	0.78 ± 0.15	0.09 ± 0.02
Root NRA (μmol nitrite/g fw/h)	0	0.33 ± 0.14	0.44 ± 0.06	0.67 ± 0.17
Shoot NRA (μmol nitrite/g fw/h)	0	0	0.021 ± 0.005	0.045 ± 0.029

Table 2 Effect of different rates of applied nitrogen (N) as nitrate (NO_3^-) on shoot dry weight (DW) and N content of gorse and wheat and the proportion of total plant N derived from the atmosphere (% Ndfa) for gorse. Pooled data for Experiments 2 and 3. Variability shown is standard error of the mean. n=8.

	Applied N (g N/m^2)			
	0	5	10	20
DW gorse (g)	1.08 ± 0.12	1.17 ± 0.10	1.22 ± 0.14	1.23 ± 0.20
N content gorse (% DW)	2.26 ± 0.10	2.41 ± 0.08	2.45 ± 0.10	2.44 ± 0.11
% Ndfa	96.6± 0.8	80.8 ± 4.0	37.5 ± 2.1	24.5 ± 4.8
DW wheat (g)	1.44 ± 0.08	4.32 ± 0.11	4.89 ± 0.24	5.79 ± 0.33
N content wheat (% DW)	1.37 ± 0.06	2.10 ± 0.07	2,56 ± 0.07	3.84 ± 0.16

Nitrate reductase is the first enzyme in the pathway of NO_3^- assimilation in plants and for most plant species is a substrate (NO_3^-) induced enzyme (Bungard *et al.* 1999; Andrews *et al.* 2013). This increased tissue NRA indicates greater NO_3^- assimilation.

The finding that gorse DW and N content were unaffected by the rate of NO_3^- application in Experiment 1 suggests that either gorse seedlings did not utilise the applied NO_3^- or that N_2 fixation decreased and NO_3^- assimilation increased with increasing NO_3^- supply. Results from the ARA and NRA assays indicate that NO_3^- assimilation increased and this was tested in Experiments 2 and 3. In Experiments 2 and 3, shoot DW and N content for gorse changed little with increased N supply (Table 2). These results are consistent with the similar response of total plant DW and N content of gorse in Experiment 1. In contrast, shoot DW and N content of wheat increased with increased N supply (Table 2). On high N supply, wheat showed substantially greater growth and N content than gorse indicating that substantial N was available for uptake. The ^{15}N analysis in Experiments 2 and 3 strongly support the results from the NRA and ARA assays in Experiment 1 and showed that the proportion of N obtained from the substrate increased with increased applied NO_3^- throughout with only 24.5 ± 4.8 %Ndfa at 20 g NO_3^--N/m^2 (200 kg N/ha equivalent) (Table 2). It is concluded that gorse shows a facultative N_2 fixation strategy.

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