

PART OF A HIGHLIGHT ON CLONAL PLANT ECOLOGY

Physiological integration modifies $\delta^{15}\text{N}$ in the clonal plant *Fragaria vesca*, suggesting preferential transport of nitrogen to water-stressed offspring

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Received: 23 December 2013 Returned for revision: 7 February 2014 Accepted: 5 March 2014 Published electronically: 25 April 2014

• **Background and Aims** One of the most striking attributes of clonal plants is their capacity for physiological integration, which enables movement of essential resources between connected ramets. This study investigated the capacity of physiological integration to buffer differences in resource availability experienced by ramets of the clonal wild strawberry plant, *Fragaria vesca*. Specifically, a study was made of the responses of connected and severed offspring ramets growing in environments with different water availability conditions (well watered or water stressed) and nitrogen forms (nitrate or ammonium).

• **Methods** The experimental design consisted of three factors, ‘integration’ (connected, severed) ‘water status’ (well watered, water stressed) and ‘nitrogen form’ (nitrate, ammonium), applied in a pot experiment. The effects of physiological integration were studied by analysing photochemical efficiency, leaf spectral reflectance, photosynthesis and carbon and nitrogen isotope discrimination, the last of which has been neglected in previous studies.

• **Key Results** Physiological integration buffered the stress caused by water deprivation. As a consequence, survival was improved in water-stressed offspring ramets that remained connected to their parent plants. The nitrogen isotope composition ($\delta^{15}\text{N}$) values in the connected water-stressed ramets were similar to those in ramets in the ammonium treatment; however, $\delta^{15}\text{N}$ values in connected well-watered ramets were similar to those in the nitrate treatment. The results also demonstrated the benefit of integration for offspring ramets in terms of photochemical activity and photosynthesis.

• **Conclusions** This is the first study in which carbon and nitrogen isotopic discrimination has been used to detect physiological integration in clonal plants. The results for nitrogen isotope composition represent the first evidence of preferential transport of a specific form of nitrogen to compensate for stressful conditions experienced by a member clone. Water consumption was lower in plants supplied with ammonium than in plants supplied with nitrate, and therefore preferential transport of ammonium from parents to water-stressed offspring could potentially optimize the water use of the whole clone.

Key words: Clonal plant ecology, clonal integration, environmental heterogeneity, *Fragaria vesca*, wild strawberry, isotope discrimination, nitrogen forms, photosynthesis, water-use efficiency.

INTRODUCTION

Natural habitats exhibit spatially heterogeneous distribution of essential resources (Lechowicz and Bell, 1991; Caldwell and Percy, 1994). This heterogeneity may be important in many plant processes, and may influence plant population structure and community dynamics (Hartgerink and Bazzaz, 1984; Price and Marshall, 1999; Day *et al.*, 2003; Wijesinghe *et al.*, 2005). Clonal reproduction results in extensive structures that occupy large areas and that are almost bound to experience spatial heterogeneity (Oborny and Cain, 1997; Hutchings *et al.*, 2004). Thus, clonal plants in particular are suitable models for studying the responses of plants to spatial environmental heterogeneity.

One of the most striking attributes of clonal plants is their capacity for physiological integration. This enables the movement of essential resources between connected ramets (Pitelka and Ashmun, 1985), allowing clonal plants to colonize unfavourable territories that otherwise would remain unexploited (Hartnett and Bazzaz, 1983; Salzman and Parker, 1985; Jónsdóttir and Watson, 1997; Saitoh *et al.*, 2002; Roiloa and Retuerto, 2006a).

Here we investigated the capacity of physiological integration to buffer differences in resource availability experienced by connected ramets of the clonal plant *Fragaria vesca*. We studied the response of connected (physiological integration allowed) and severed (physiological integration prevented) offspring ramets growing in environments with different water availability conditions (well watered or water stressed) and nitrogen forms (nitrate or ammonium). The responses of integrated ramets to heterogeneous distribution of nutrients have been studied extensively (Slade and Hutchings, 1987; Wijesinghe and Hutchings, 1999; Saitoh *et al.*, 2006; Hutchings and Wijesinghe, 2008; D’Hertefeldt *et al.*, 2011). However, to our knowledge only one study has attempted to determine the effects of physiological integration on ramets supplied with different forms of nitrogen (Jónsdóttir and Callaghan, 1990). The form of nitrogen absorbed by plants has been shown to affect plant growth (Claussen and Lenz, 1999; Tabatabaei *et al.*, 2006; Guo *et al.*, 2007). Nitrate and ammonium are the predominant inorganic nitrogen forms absorbed and metabolized by plant roots (Marschner, 1995). Ammonium uptake by roots appears to be a passive process, in

contrast to both active and passive processes involved in the acquisition of nitrate (Higinbotham, 1973; Breteler and Hänisch ten Cate, 1980). Uptake of ammonium generally occurs at a higher rate than that of nitrate (Lee and Stewart, 1978; Marty *et al.*, 2009; Mota *et al.*, 2011; Gherardi *et al.*, 2013; Lian *et al.*, 2012; Yang *et al.*, 2013). For most species, optimal growth is generally achieved when both nitrogen forms are available. However, the preferential form of nitrogen varies with species and environmental conditions (Errebhi and Wilcox, 1990). For example, although *Fragaria ananassa* grows better with nitrate than with ammonium as the sole nitrogen form (Darnell and Stutte, 2001), the growth rate is higher when both nitrate and ammonium are available (Ganmore-Neumann and Kafkafi, 1985). Previous studies have demonstrated low water consumption by plants supplied with ammonium compared with those supplied with nitrate (Guo *et al.*, 2007). Therefore, we also aimed to determine how ammonium and nitrate affect plants growing under different conditions of water availability and how this may be modified by physiological integration.

We studied the effect of clonal integration at morphological and physiological levels. A number of studies have attempted to determine the effects of clonal integration on physiological traits, such as photosynthetic rates and photochemical efficiencies. Some studies have reported how clonal integration may allow, by a mechanism of feedback regulation, an enhancement of the photosynthetic efficiency of parents connected to daughter ramets growing in stressful conditions (Hartnett and Bazzaz, 1983; Roiloa and Retuerto, 2006a, b; Roiloa *et al.*, 2007; Liu *et al.*, 2008; Zhang *et al.*, 2009). The present study aims to improve our understanding of the effects of clonal integration on photosynthetic process. Although previous studies have used single isotopes as labels or tracers to demonstrate resource translocation in clonal plants (Derner and Briske, 1998; D'Hertefeldt and Jonsdottir, 1999; D'Hertefeldt and Falkengren-Grerup, 2002; Hellström *et al.*, 2006; Saitoh *et al.*, 2006), our research is the first that uses stable isotope ratios to assess how physiological integration affects the way in which clonal plants use and acquire resources (water and nitrogen). Isotope discrimination techniques have been applied extensively in plant ecological research during the last few decades (Michener and Lajtha, 2007). However, no research has been carried out to determine how physiological integration affects isotope carbon discrimination and isotopic nitrogen composition in clonal plants. Due to its biochemical properties, the Rubisco enzyme discriminates against $^{13}\text{CO}_2$ more than it does against $^{12}\text{CO}_2$ (Farquhar *et al.*, 1989). The lower the concentration of CO_2 (C_i) at the active site of Rubisco, the lower the discrimination against the heavy isotope ($\Delta^{13}\text{C}$). The value of C_i reflects the balance between stomatal conductance and consequently the availability of water and the photosynthetic requirement for carbon dioxide. Thus, a low C_i and the consequent low $\Delta^{13}\text{C}$ may be the result of either a high photosynthetic rate or, more commonly, low stomatal conductance, which would result in greater integrated water-use efficiency (Körner *et al.*, 1988; Farquhar *et al.*, 1989). The $^{15}\text{N}/^{14}\text{N}$ ratio ($\delta^{15}\text{N}$) has been proposed as an integrative trait to assess stress tolerance in plants (Robinson 2001). Plant and soil $\delta^{15}\text{N}$ variation is primarily due to physiological and biogeochemical processes involved in the N cycle. Thus, the processes of N mineralization, nitrification, denitrification or NH_3 volatilization discriminate against the heaviest isotope of nitrogen, so that that the different nitrogen

forms have distinctive $\delta^{15}\text{N}$ signatures (Handley and Raven, 1992; Robinson, 2001).

Movement of water and nitrogen between integrated ramets enables the clone to buffer environmental heterogeneity (Slade and Hutchings, 1987; Roiloa and Retuerto, 2007; Mao *et al.*, 2009; D'Hertefeldt *et al.*, 2011). Consequently, we expect similar carbon and nitrogen isotopic compositions (as indicators of water availability and nitrogen form, respectively) within the interconnected modules of the clone, despite differences in resource availability in the patches.

We tested the following specific hypotheses in the present study. (1) Physiological integration will buffer the stress induced by water deprivation. We predicted that water-stressed conditions in offspring ramets would be ameliorated by the movement of water from well-watered parents. As a result, we expected less restrictive water use (i.e. higher carbon discrimination, according to Farquhar *et al.*, 1989) and higher rates of photosynthesis, growth and survival in connected than in severed water-stressed offspring ramets. (2) Physiological integration will allow the transport of N as nitrate or ammonium from parents to offspring ramets, depending on whether the offspring ramets are growing in soils lacking nitrate or ammonium, respectively. The form of nitrogen (ammonium or nitrate) taken up by plants will contribute to the determination of the nitrogen isotopic composition ($\delta^{15}\text{N}$) of their tissues. Given that nitrate-fed plants tend to be enriched in the heaviest N isotope, whereas ammonium fed plants are depleted (Evans, 2001; Kahmen *et al.*, 2008; Ariz *et al.*, 2011), we expected that integration would bring about changes in $\delta^{15}\text{N}$, leading to similar values in offspring ramets, regardless of whether the ramets were grown with ammonium or nitrate as the available nitrogen form. (3) Since water consumption is lower in plants supplied with ammonium than in plants supplied with nitrate (Høgh-Jensen and Schjoerring, 1997; Guo *et al.*, 2007) and considering that ammonium uptake generally occurs at a higher rate than nitrate uptake (Lee and Stewart, 1978), we expected lower $\delta^{15}\text{N}$ in integrated ramets than in disconnected ramets due to preferential transport of ammonium to offspring ramets under water-stressed conditions. As consequence, if, as reported in previous studies (Kahmen *et al.*, 2008), ammonium uptake leads to a decrease in $\delta^{15}\text{N}$, we would expect a lower $\delta^{15}\text{N}$ in connected water-stressed offspring than in connected well-watered offspring.

MATERIALS AND METHODS

Plant material

The study was carried out with the clonal wild strawberry plant *Fragaria vesca*, which is a perennial, evergreen herb of the Rosaceae family and is widely distributed in Europe, North America and Asia, where it inhabits a broad range of habitats. The habitat range of *F. vesca* includes sites such as stabilized scree, quarry and mine spoils, wastelands and pastures. It is frequent in open woodland and scrub and present in hedgerows, but it is absent from wetlands and arable land. This wide-ranging species is associated with vegetation characteristics of relatively infertile conditions. It is absent from highly disturbed or productive communities (Grime *et al.*, 1996). *Fragaria vesca* has a high capacity for clonal reproduction by stolons that produce above-ground ramets at every other node. Clonal reproduction

enables *F. vesca* to form fairly large clonal systems that are likely to experience small-scale environmental heterogeneity. A single genet can spread over more than 2 m² in 1 year (S. R. Roiloa, pers. observations). Clones of *F. vesca* are integrated physiologically through vascular connections that allow translocation of substances between the connected ramets.

Plant material for the study was collected from three different sites in a forest understorey located 10 km south of Santiago de Compostela (north-west Spain) and vegetatively propagated in an open-end greenhouse for several months. The sites were at least 50 m from one another, so that it was assumed that each set of plants was derived from different genotypes. Previous studies with the congener stoloniferous *Fragaria chiloensis* have shown that plants more than 10 m apart are almost certain to belong to different clones (Alpert *et al.*, 1993). To avoid possible confounding effects of genotypes, the plants were assigned to the treatments at random.

Experimental design

The experimental design consisted of three crossed factors: 'integration' (connected, severed), 'water status' (well watered, water stressed) and 'nitrogen form' (nitrate, ammonium) (Fig. 1). Eighty pairs of ramets (parent and offspring ramets) were selected for uniformity of size, from the plant stock. The ramets of each pair were rooted individually in 0.75-L plastic pots, filled with potting compost for the parent ramet and with washed sand for the offspring ramet. Washed sand was used to enable close control of nutrient availability.

Offspring ramets were subjected to each of two different water availability conditions: well watered and water stressed. Soil water content was monitored with a soil moisture meter every 3 days throughout the experiment (Moisture Meter HH2; Delta-T Devices, Cambridge, UK). The volumetric soil moisture

content was determined as the ratio between the volume of water present and the total volume of the sample. This is a dimensionless parameter, expressed as a percentage (% vol). Well-watered offspring ramets were maintained at field capacity by watering them with as much water as necessary to maintain an average volumetric soil water content of 20.7 % vol. We have previously determined that the water capacity for the pots used corresponds to a volumetric soil moisture content of 22 %. Water-stressed offspring ramets were subjected throughout the experiment to consecutive cycles of water deprivation by withholding water, but preventing the soil water content from decreasing below 8 %, to avoid wilting of the water-stressed plants. During the water deprivation cycles, water-stressed plants received only the amount of water required to maintain an average volumetric soil moisture content of 8.8 % vol. At the end of each 2-week cycle of water deprivation we brought water-stressed plants to field capacity. At the end of the experiment, water-stressed plants had received 43 % of the water added to well-watered ramets. The duration of water deprivation cycles was 2 weeks. Soil moisture was significantly higher in the well-watered than in the water-stressed treatment (ANOVA: $F_{1,78} = 432.38$, $P < 0.001$). Parent ramets were maintained at field capacity during the experiment.

The nitrogen form treatment was applied by adding a nutrient solution containing nitrate or ammonium to the offspring ramets as the sole nitrogen form (modified from Thomas *et al.*, 1979) (Table 1). Replacement of nitrate (anion) by ammonium (cation) in a nutrient solution leads to important changes in the cation/anion balance. To maintain the cation/anion balance as equilibrated as possible we slightly modified the concentration of additional ions (PO_4^{3-} , K^+ , Ca^{2+} and SO_4^{2-}) (Table 1). The nitrate and ammonium solutions contained identical amounts of N and provided an adequate nutrient mixture for plant growth. A nutrient solution was applied to the plants every 2 weeks. To avoid nutrient build-up, each ramet was

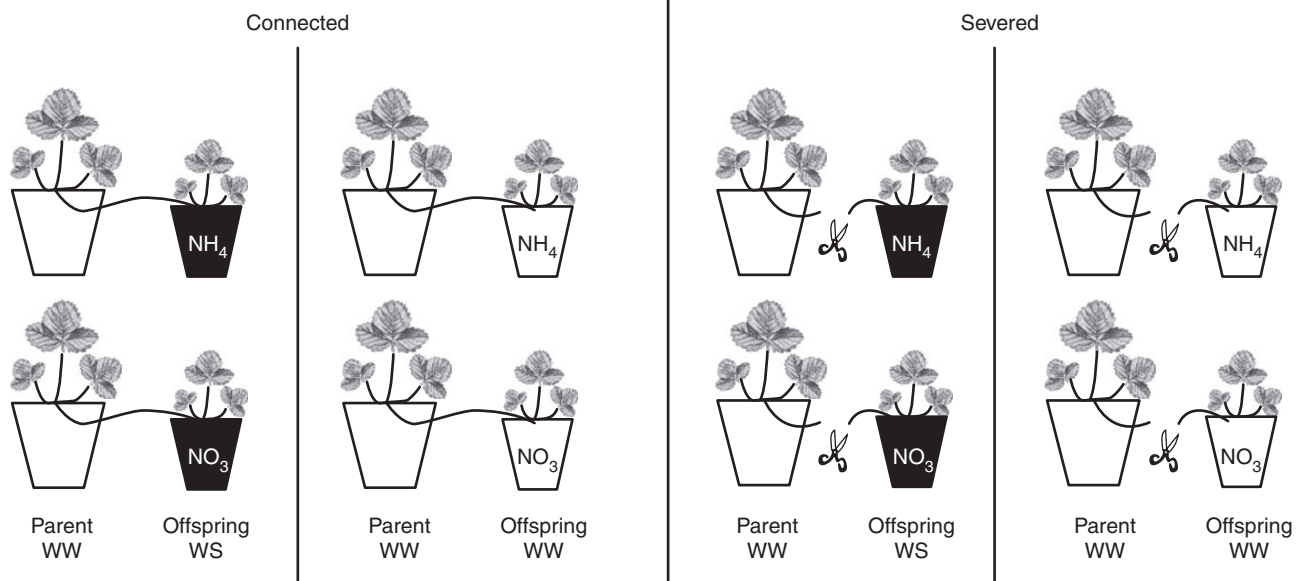


FIG. 1. Schematic representation of the experimental treatments showing the N form supplied (nitrate, NO₃; ammonium, NH₄) and the water treatments (white pots for well watered, WW; black pots for water stressed, WS) applied to pairs of connected and severed ramets. Each treatment was replicated ten times.

always watered with 200 mL of solution, which was sufficient to ensure that some solution drained freely from the pot. Parent ramets were grown in a potting compost substrate (CompoSana, Compo Iberia, Barcelona, Spain) containing all main nutrients and trace elements required for optimal growth of plants. Nitrate and ammonium contents in the potting compost of the parents were determined using the Kjeldahl method (Kjeldahl Distiller Pro-Nitro M, JP Selecta, Barcelona, Spain), and were found to be 1523 p.p.m. of NO_3^- and 141 p.p.m. of NH_4^+ . The presence of nitrate and ammonium in the potting compost allows the potential transport of the two nitrogen forms from the parents to their connected offspring ramets.

At the beginning of the experiment, in order to determine the effect of physiological integration, the stolon connections were severed in half of the ramet pairs, selected at random. The new ramets produced during the experiment remained connected to their parents but were prevented from rooting.

The experiment was performed in a greenhouse under a natural day/night light cycle. After 2 weeks to allow plant establishment, the treatments began on 23 April 2010 and continued for 60 days. Each treatment was replicated ten times.

Measurements

Chlorophyll fluorescence. Chlorophyll fluorescence parameters were measured by the saturation pulse method (Schreiber et al., 1998) with a portable fluorometer (MINI-PAM photosynthesis yield analyser; Walz, Effeltrich, Germany) 15, 30, 45 and 60 days after treatment application. A pulse of saturating light ($>4000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, 0.8 s pulse length, actinic white light) was applied through an optical fibre at an angle of 60° relative to the sample and a distance of 12 mm from the leaf. Measurements were made on the upper surface of the youngest fully expanded leaf of each offspring ramet. The maximum quantum yield of photosystem II (PSII) and the

TABLE 1. Salts (g L^{-1}) and ions (mM) in the nitrate and ammonium solutions

NO_3^- solution				NH_4^+ solution			
Salt	g L^{-1}	Ion	mM	Salt	g L^{-1}	Ion	mM
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	0.8	NH_4^+	0	$(\text{NH}_4)_2\text{SO}_4$	0.5	NH_4^+	7.46
KH_2PO_4	0.8	NO_3^-	6.78	K_2HPO_4	0.3	NO_3^-	0
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.75	PO_4^{3-}	5.88	KH_2PO_4	0.46	PO_4^{3-}	5.1
$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$	0.645	K^+	5.88	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.75	K^+	6.82
Fe-EDTA	0.042	Ca^{2+}	7.14	$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$	0.89	Ca^{2+}	5.17
Trace elements	0.5	Mg^{2+}	3.04	Fe-EDTA	0.042	Mg^{2+}	3.04
		SO_4^{2-}	6.79	Trace elements	0.5	SO_4^{2-}	11.94

TABLE 2. Results of three-way repeated-measures analysis of variance, with 'integration', 'nitrogen form' and 'water status' as between-subject effects, for differences in chlorophyll fluorescence parameters [maximum (F_v/F_m) and effective (Φ_{PSII}) quantum yield of photosystem II] and spectral reflectance [photochemical reflectance index (PRI) and chlorophyll content index (CHL)] between offspring ramets

	df	F_v/F_m		Φ_{PSII}		PRI		CHL	
		F	P	F	P	F	P	F	P
Between-subject effect									
Integration	1	0.284	0.596	21.169	<0.001	23.283	<0.001	23.143	<0.001
Nitrogen	1	0.210	0.649	1.212	0.276	1.075	0.305	1.060	0.308
Water	1	6.563	0.013	4.943	0.030	4.076	0.048	5.035	0.029
Integration × nitrogen	1	1.597	0.212	0.331	0.568	0.951	0.334	0.978	0.327
Integration × water	1	2.042	0.159	0.199	0.657	1.168	0.285	1.019	0.317
Nitrogen × water	1	0.033	0.856	0.075	0.785	0.603	0.441	1.113	0.296
Integration × nitrogen × water	1	0.619	0.435	0.263	0.610	0.004	0.947	0.058	0.810
Error	54								
Within-subject effect									
Time	3	9.616	<0.001	27.183	<0.001	21.113	<0.001	42.895	<0.001
Integration × time	3	2.069	0.106	3.161	0.026	0.346	0.792	0.740	0.530
Nitrogen × time	3	0.385	0.764	0.417	0.741	0.213	0.888	0.448	0.719
Water × time	3	1.339	0.264	1.725	0.164	5.044	0.002	10.872	<0.001
Integration × nitrogen × time	3	0.222	0.881	1.826	0.144	0.278	0.841	0.539	0.656
Integration × water × time	3	0.999	0.395	0.947	0.419	1.736	0.162	0.535	0.659
Nitrogen × water × time	3	0.498	0.684	0.371	0.774	2.236	0.086	0.287	0.835
Integration × nitrogen × water × time	3	0.304	0.822	0.215	0.886	0.886	0.450	0.960	0.413
Error	162								

See Fig. 2 for data.

Significant effects ($P < 0.05$) are shown in bold type.

effective quantum yield of PSII (Φ_{PSII}) were determined. We calculated the maximum quantum yield of photosystem II PSII as the ratio $F_v/F_m = (F_m - F_0)/F_m$ (Bolh ar-Nordenkamp *et al.*, 1989), where F_0 and F_m are the minimal and maximal fluorescence yields of a dark-adapted sample, respectively, with all PSII reaction centres fully open (i.e. all primary acceptors oxidized). This parameter was measured after dark adaptation for 30 min, which is considered sufficient time to enable opening of all PSII reaction centres in *F. vesca* plants (Roiloa and Retuerto, 2005). The F_v/F_m ratio provides an estimate of the efficiency of excitation energy capture by open PSII reaction centres (Butler and Kitajima, 1975). We calculated Φ_{PSII} as $(F'_m - F_i)/F'_m$ (Genty *et al.*, 1989), where F'_m is the maximal fluorescence yield reached in a pulse of saturating light with an illuminated sample and F_i is the fluorescence yield of the leaf at a given photosynthetic photon flux density. This parameter was measured under artificial light conditions of $692.6 \pm 113.0 \mu\text{mol m}^{-2} \text{s}^{-1}$ (mean \pm s.e., $n = 320$) provided by an auxiliary lamp (External Halogen Lamp 2050-HB; Walz). Φ_{PSII} is a measure of the fraction of the light absorbed by chlorophyll that is photochemically converted into PSII (Maxwell and Johnson, 2000).

Leaf spectral reflectance. Immediately after the chlorophyll fluorescence measurements, the spectral reflectance parameters were determined in offspring ramets with a portable spectrometer (UniSpec Spectral Analysis System; PP Systems, Haverhill, MA, USA). We measured the photochemical reflectance index (PRI) and the chlorophyll content index (CHL) in the same leaves as those used to measure chlorophyll fluorescence parameters. The PRI was calculated as $(R_{531} - R_{570})/(R_{531} + R_{570})$, where R refers to reflectance and the subscripts to a specific spectral wavelength in nanometres. The 531 nm wavelength is sensitive to changes in the epoxidation state of the xanthophyll cycle, and 570 nm is a reference wavelength unaffected by xanthophyll activity (Guo and Trotter, 2004). The PRI is significantly correlated with both net CO_2 uptake and the photosynthetic radiation use efficiency ($\text{mol CO}_2 \text{ mol}^{-1} \text{ photons}$) (Pe uelas *et al.*, 1995; Gamon *et al.*, 1997). It is also thought to be correlated with the levels of de-epoxidized (photoprotective) xanthophyll cycle pigments (Sims and Gamon, 2002; Stylinski *et al.*, 2002). The CHL, calculated as R_{750}/R_{700} , is correlated with the chlorophyll content of leaves (Wood *et al.*, 1993; Lichtenthaler *et al.*, 1996).

Gas exchange. At the end of the experiment, leaf gas exchange measurements were made in offspring ramets, with a portable photosynthesis system (LI-6400XT; Li-Cor, Lincoln, NE, USA) at a leaf temperature of 27°C and at the natural irradiance at midday (photosynthetically active radiation $605.9 \pm 1.8 \mu\text{mol m}^{-2} \text{s}^{-1}$, mean \pm s.e., $n = 58$). Photosynthetic carbon assimilation, stomatal conductance and the intercellular CO_2 concentration were also determined. The small size of the leaves prevented us from recording gas exchange parameters in four offspring ramets.

Carbon and nitrogen isotope discrimination and leaf content. At the end of the experiment, two new leaves that had grown during the experiment were harvested from each treated offspring ramet and bulked for determination of carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) isotopic compositions. Sampled leaves were cleaned of organic debris, oven-dried at 70°C for 48 h, then ball-milled to a fine homogenized powder to pass through a 40-mesh screen.

Determinations of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ for each offspring ramet were made with an isotope ratio mass spectrometer (Delta Plus; Finnigan MAT, San Jose, CA, USA). The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of samples were calculated as $\delta(\text{‰}) = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000$, where R is the ratio of heavy to light isotope, R_{sample} is the ratio in the sample and R_{standard} is the

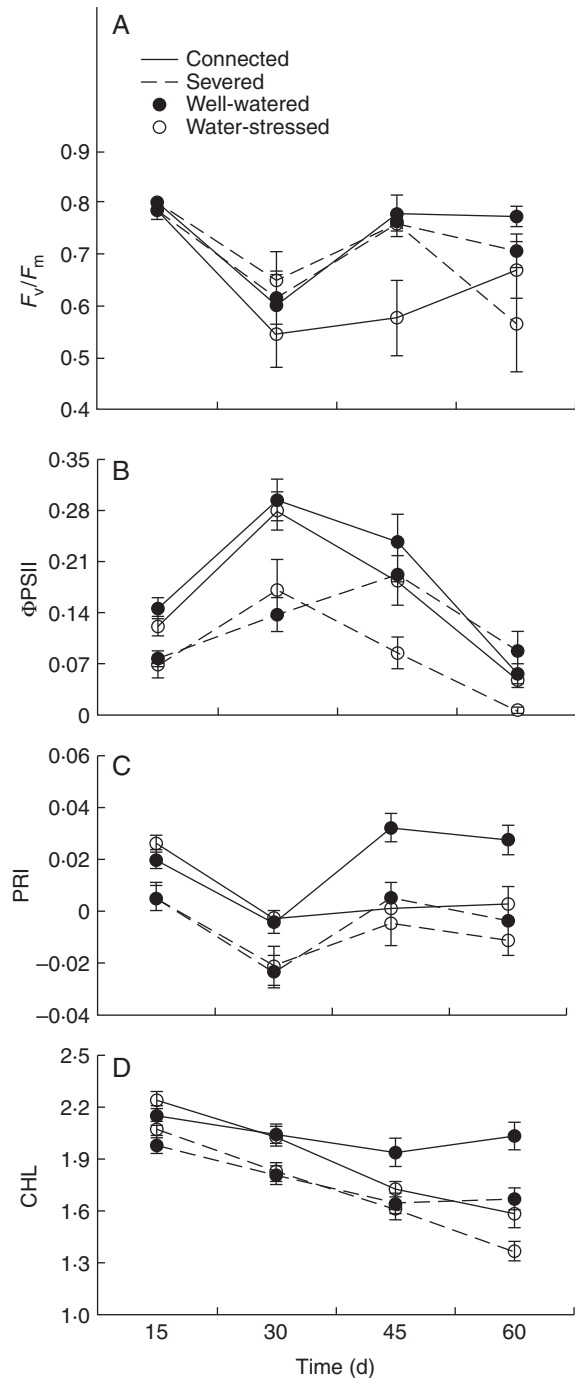


FIG. 2. Time course of mean values (\pm s.e.) of chlorophyll fluorescence parameters F_v/F_m (A) and Φ_{PSII} (B) and the spectral reflectance parameters photochemical reflectance index (PRI) (C) and chlorophyll content index (CHL) (D) for connected (solid lines) and severed (dashed lines) offspring ramets in well-watered (closed symbols) and water-stressed (open symbols) treatments. See Table 2 for ANOVA parameters.

ratio in the Pee Dee Belemnite carbonate standard. We calculated $^{13}\text{C}/^{12}\text{C}$ discrimination ($\Delta^{13}\text{C}$) as $\Delta^{13}\text{C} (\text{‰}) = [(\delta^{13}\text{C}_{\text{air}} - \delta^{13}\text{C}_{\text{sample}})/(1000 + \delta^{13}\text{C}_{\text{sample}})] \times 1000$. Under the well-mixed air conditions of the study, we assumed a $\delta^{13}\text{C}$ value for atmospheric CO_2 of -8.05‰ (Ferrio et al., 2005). $\Delta^{13}\text{C}$ has been used as an indicator of plant ecophysiological processes such as leaf conductance, water-use efficiency and photosynthetic capacity (Körner et al., 1988; Farquhar et al., 1989; Ehleringer et al., 1993). The plant $\delta^{15}\text{N}$ value provides information about the source, absorption and assimilation of nitrogen in plants (Evans, 2001). Carbon and nitrogen contents (percentage dry mass) were determined in the same samples as those in which isotopic composition was measured, with an elemental analyser (EA 1108 CHNS/O; Carlo Erba Instruments, Milan, Italy).

Growth and survival. At the end of the experiment, offspring ramets were harvested separately, divided into above- and below-ground structures and dried to constant weight at 60°C to determine root dry mass, total dry mass and the proportional biomass allocated to roots (root dry mass ratio = root dry mass/total dry mass). The newly produced stolons and new ramets were assigned to the above-ground biomass of the ramets producing them. Mortality rates of offspring ramets were determined every week.

Statistical analyses

Total mass and root mass were \log_{10} -transformed and root mass ratio was square root-transformed to meet the requirements of normality and homoscedasticity for ANOVA.

Differences between treatments for growth, isotopic composition, carbon and nitrogen contents and gas exchange were evaluated by three-way ANOVA, with ‘integration’, ‘nitrogen form’ and ‘water status’ as the main factors. Two additional two-way ANOVAs were conducted separately for connected and severed ramets in order to detect the effects of ‘nitrogen form’ and ‘water status’ on isotopic composition. Differences in chlorophyll fluorescence and spectral reflectance parameters were analysed by repeated measures three-way analysis of variance (ANCOVA), with ‘integration’, ‘nitrogen form’ and ‘water status’ as between-subject effects and ‘time’ as the within-subject effect. Survivorship during the experiment was analysed

using Kaplan–Meier analysis with the log rank (Mantel–Cox) test (Kaplan and Meier, 1958).

Mortality reduced the number of replicates used in the different analyses, as indicated by the error degrees of freedom. Differences were considered significant at $P < 0.05$. Statistical tests were performed with SPSS 18.0 (SPSS, Chicago, IL, USA).

RESULTS

Chlorophyll fluorescence and leaf spectral reflectance

In water-stressed ramets there were significant decreases, relative to well-watered ramets, in maximum quantum yield of PSII (F_v/F_m) and ΦPSII , PRI and CHL, regardless of whether the ramets were connected to the parent ramet or not (Table 2, Fig. 2). Integration significantly affected chlorophyll fluorescence and leaf reflectance parameters. The ΦPSII , PRI and CHL values were significantly higher in connected offspring ramets than in severed offspring ramets (Table 2, Fig. 2). No significant effects of the form of nitrogen or of the interactions of the studied factors were detected for any of the chlorophyll fluorescence and spectral reflectance variables (Table 2).

Gas exchange

Severed and water-stressed offspring ramets experienced a significant reduction in photosynthetic carbon assimilation and stomatal conductance relative to connected and well-watered ramets, respectively (Table 3, Fig. 3). No significant effects were detected for the nitrogen form or for the interactions of the studied factors (Table 3).

Carbon and nitrogen isotope discrimination and leaf content

Nitrogen isotopic composition ($\delta^{15}\text{N}$) was significantly affected by the nitrogen form. The value of $\delta^{15}\text{N}$ was significantly lower in offspring ramets supplied with ammonium than in offspring ramets supplied with nitrate (Table 4, Fig. 4A). The interaction between integration and water was significant for $\delta^{15}\text{N}$. The $\delta^{15}\text{N}$ values of severed ramets were similar in the well-watered and water-stressed treatments. However, for connected ramets, $\delta^{15}\text{N}$ was lower in the water-stressed treatment than in the well-watered treatment (Table 4, Fig. 4B). This result was

TABLE 3. Three-way ANOVA for analyses of differences in photosynthetic carbon assimilation, stomatal conductance and intercellular CO_2 concentration (C_i) of offspring ramets with ‘integration’, ‘nitrogen form’ and ‘water status’ as main factors

Effect	df	Photosynthesis		Conductance		C_i	
		F	P	F	P	F	P
Integration	1	6.731	0.012	10.831	0.002	0.335	0.566
Nitrogen	1	1.981	0.165	1.500	0.226	0.134	0.716
Water	1	15.469	<0.001	21.077	<0.001	0.302	0.585
Integration × nitrogen	1	0.179	0.674	0.204	0.653	0.055	0.815
Integration × water	1	3.211	0.079	2.772	0.102	2.277	0.138
Nitrogen × water	1	0.910	0.345	1.150	0.289	1.409	0.241
Integration × nitrogen × water	1	0.010	0.919	0.002	0.966	0.272	0.605
Error	50						

See Fig. 3 for data.

Significant effects ($P < 0.05$) are shown in bold type.

supported by the separate two-way ANOVA conducted for connected and severed ramets. In this way, we detected a significant effect of the interaction between nitrogen form and water for the connected treatment (ANOVA, $F_{1, 33} = 4.225$, $P = 0.048$; Fig. 4C). For connected ramets, we found a significant reduction in $\delta^{15}\text{N}$ with water stress, and this reduction was especially strong for ramets growing in the nitrate treatment. However, this effect was not detected in the severed treatment (ANOVA, $F_{1, 33} = 0.003$, $P = 0.960$; Fig. 4D). The $\delta^{15}\text{N}$ values in the connected ramets in the water-stressed treatment were similar to those in ramets in the ammonium treatment. However, $\delta^{15}\text{N}$ values in connected well-watered ramets were similar to those in the nitrate treatment (Fig. 4).

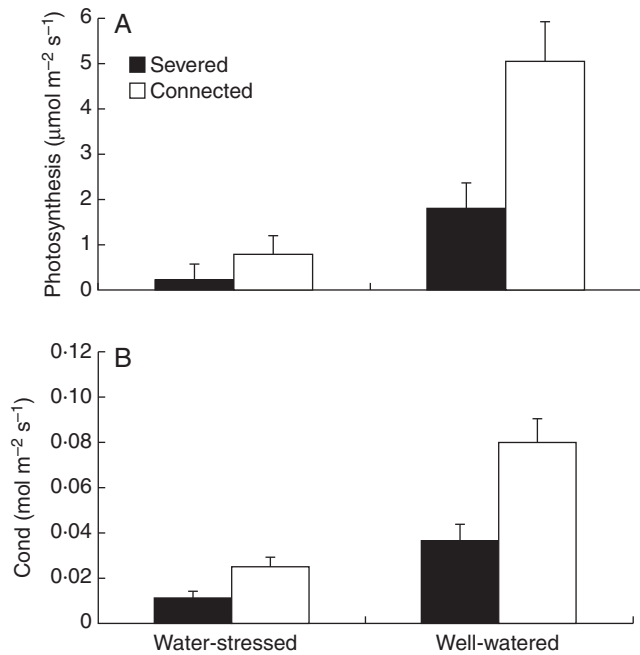


FIG. 3 Mean (\pm s.e.) photosynthetic carbon assimilation (A) and stomatal conductance (B) of severed and connected offspring ramets (as indicated in the key) in the water-stressed and well-watered treatments. See Table 3 for ANOVA parameters.

Water status had a significant effect on $^{13}\text{C}/^{12}\text{C}$ discrimination ($\Delta^{13}\text{C}$), leaf nitrogen content (%N) and leaf carbon content (%C). The value of $\Delta^{13}\text{C}$ was lower in well-watered ramets than in water-stressed ramets (Table 4, Fig. 5A). Both %N and %C were significantly higher in well-watered ramets than in water-stressed ramets (Table 4, Fig. 5B, C). The effect of integration on %N and %C depended on the water treatment (Table 4). Thus, integration significantly increased %N and decreased %C in well-watered offspring ramets but not in the water-stressed ramets (Table 4, Fig. 5B, C).

Growth and survival

Root mass, root mass ratio and total dry mass of water-stressed offspring ramets were significantly reduced relative to well-watered offspring ramets (Table 5, Fig. 6). Connection increased the survival of offspring ramets growing in water-stressed conditions ($\chi^2 = 23.955$, $P < 0.001$) (Fig. 7).

DISCUSSION

The increased survival of water-stressed offspring ramets connected to parents supports our first hypothesis that physiological integration would buffer the stress caused by water deprivation. Previous studies have shown that offspring ramets growing in unfavourable conditions may be supported by the translocation of resources from parent ramets established in favourable patches, thus increasing their growth and survival (Hartnett and Bazzaz, 1983; Slade and Hutchings, 1987; Jónsdóttir and Watson, 1997; Roiloa and Retuerto, 2006a; Saitoh et al., 2006).

Although most of the literature on clonal plant ecology has focused on morphological responses (growth, biomass allocation ratios, stolon length, etc.), the present study demonstrates that the effects of integration can also be detected at a physiological level. The findings of the present study demonstrate the benefit of integration for the offspring ramets in terms of photochemical activity and photosynthesis, regardless of water status and the nitrogen form supplied. Similar benefits in photosynthetic traits have also been reported for offspring of *F. vesca* growing under unfavourable conditions (Roiloa and Retuerto, 2006a;

TABLE 4. Three-way ANOVA for analyses of differences in nitrogen isotopic composition ($\delta^{15}\text{N}$), $^{13}\text{C}/^{12}\text{C}$ discrimination ($\Delta^{13}\text{C}$), leaf nitrogen content (%N) and leaf carbon content (%C) of offspring ramets with ‘integration’, ‘nitrogen form’ and ‘water status’ as main factors

Effect	df	$\delta^{15}\text{N}$		$\Delta^{13}\text{C}$		%N		%C	
		F	P	F	P	F	P	F	P
Integration	1	0.668	0.417	3.338	0.073	2.498	0.120	0.259	0.613
Nitrogen	1	46.831	<0.001	0.179	0.674	0.009	0.924	0.165	0.686
Water	1	3.615	0.063	4.690	0.035	4.016	0.050	14.700	<0.001
Integration \times nitrogen	1	3.305	0.075	0.126	0.724	0.376	0.542	1.284	0.262
Integration \times water	1	7.740	0.007	0.535	0.468	5.796	0.020	5.030	0.029
Nitrogen \times water	1	1.373	0.246	0.021	0.886	0.789	0.378	0.906	0.345
Integration \times nitrogen \times water	1	1.696	0.198	1.637	0.206	2.252	0.139	0.243	0.624
Error	54								

See Figs 4 and 5 for data. Significant effects ($P < 0.05$) are shown in bold type. df, degrees of freedom.

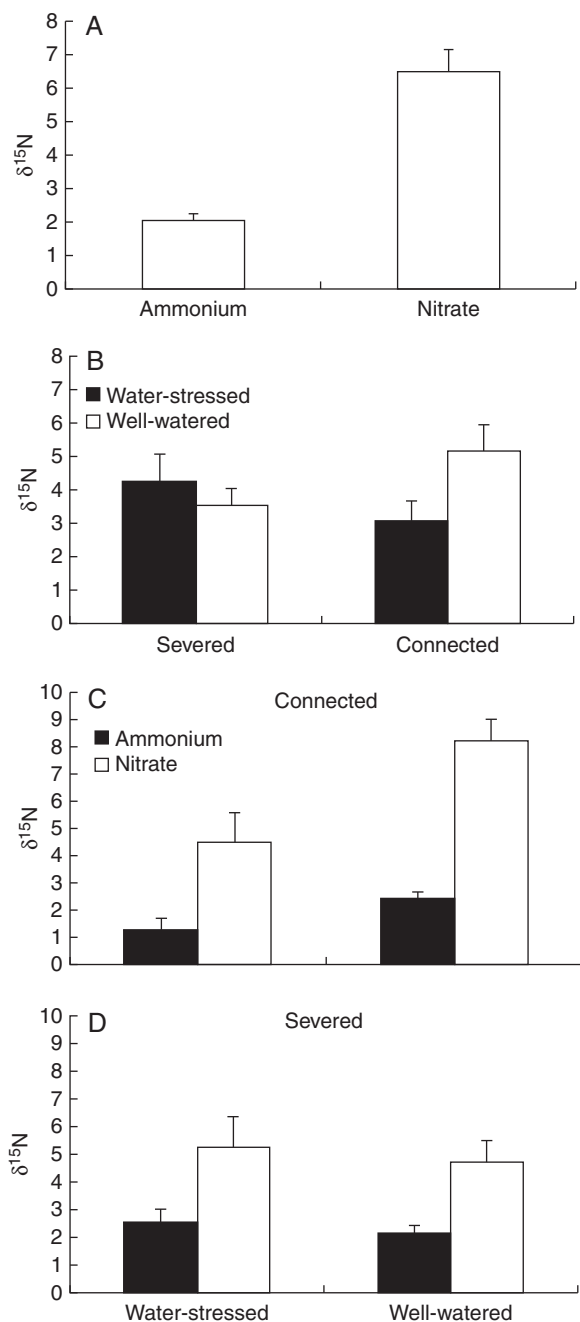


FIG. 4. Mean (\pm s.e.) nitrogen isotopic composition ($\delta^{15}\text{N}$) in offspring ramets in ammonium and nitrate treatments (A) and in connected and severed offspring ramets in water-stressed and well-watered treatments (B). (C, D) Mean (\pm s.e.) nitrogen isotopic composition ($\delta^{15}\text{N}$) in offspring ramets in water-stressed and well-watered treatments and growing in nitrate or ammonium nitrogen for connected ramets (C) and severed offspring ramets (D). See Table 4 and text for ANOVA parameters.

Roiloa and Retuerto, 2007). However, the benefits of integration in terms of photochemical activity and photosynthesis were not translated into benefits in growth. Although connected offspring produced more total dry mass than severed offspring, the differences were not significant. Considering that growth in term of dry mass is the result of the balance between photosynthesis and respiration, our results strongly suggest that integration also

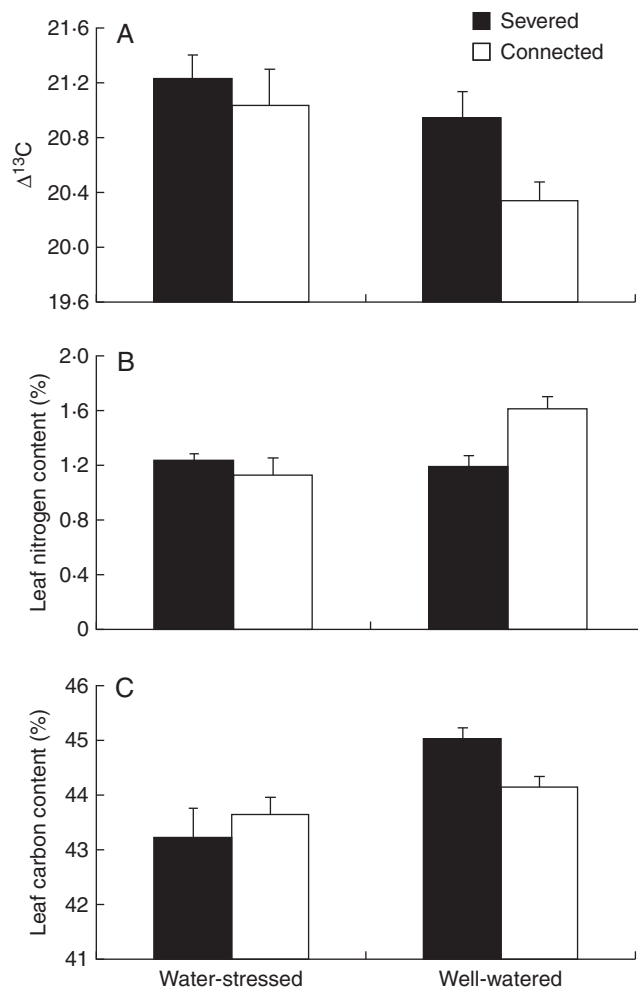


FIG. 5. Mean $^{13}\text{C}/^{12}\text{C}$ discrimination ($\Delta^{13}\text{C}$) (A), leaf nitrogen content (B) and leaf carbon content (C) of severed and connected offspring ramets (as indicated in the key) in the water-stressed and well-watered treatments. See Table 4 for ANOVA parameters.

increased respiration at a rate that cancelled out the benefits of an increased photosynthetic rate.

Contrary to our prediction, the $\Delta^{13}\text{C}$ of water-stressed ramets was not affected by integration. The $\Delta^{13}\text{C}$ value reflects the ratio of leaf internal to external CO_2 concentration and has been used as a suitable integrator of stomatal conductance, water-use efficiency and photosynthetic rate of plants (Körner *et al.*, 1988; Farquhar *et al.*, 1989; Ehleringer *et al.*, 1993). High $\Delta^{13}\text{C}$ values are associated with high stomatal conductance and consequently with high photosynthetic activity and growth. An increase in $\Delta^{13}\text{C}$ would be expected when the water availability was high (Ehleringer and Cooper, 1988; Farquhar *et al.*, 1989). Well-watered conditions will enlarge stomatal apertures and increase $\Delta^{13}\text{C}$ (Farquhar *et al.*, 1989). However, the present results showed a significant increase in $\Delta^{13}\text{C}$ in the water-stressed ramets relative to the well-watered ramets. In our study, the higher $\Delta^{13}\text{C}$ in water-stressed plants occurred together with a significant decrease in photochemical activity, photosynthetic rate, stomatal conductance and growth experienced by the water-stressed plants in the present study. According to

TABLE 5. Three-way ANOVA for analyses of differences in root biomass, root mass ratio and total biomass of offspring ramets with 'integration', 'nitrogen form' and 'water status' as main factors

Effect	df	Root biomass		Root mass ratio		Total biomass	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Integration	1	2.002	0.163	0.177	0.675	3.187	0.080
Nitrogen	1	3.752	0.058	1.322	0.255	2.255	0.139
Water	1	10.787	0.002	4.955	0.033	6.956	0.011
Integration × nitrogen	1	0.744	0.392	0.207	0.651	0.106	0.746
Integration × water	1	0.229	0.634	0.287	0.594	0.495	0.485
Nitrogen × water	1	0.071	0.791	0.444	0.508	0.600	0.442
Integration × nitrogen × water	1	0.137	0.713	2.789	0.100	2.848	0.097
Error	54						

See Fig. 6 for data.

Significant effects ($P < 0.05$) are shown in bold type.

df, degrees of freedom.

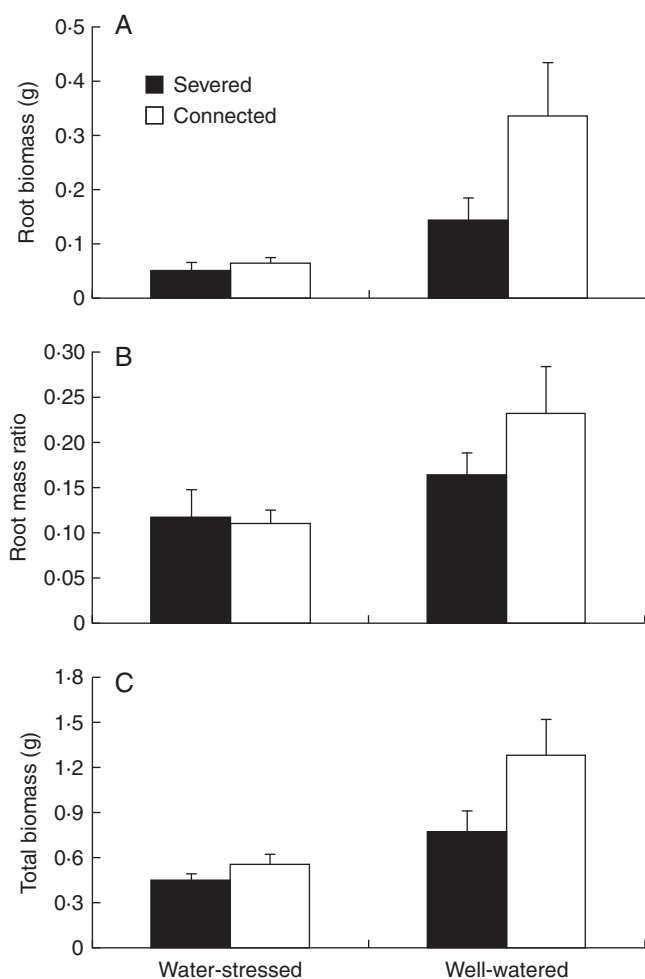


FIG. 6. Mean (\pm s.e) root dry mass (A), root mass ratio (B) and total dry mass (C) of severed and connected offspring ramets (as indicated in the key) in the water-stressed and well-watered treatments. See Table 5 for ANOVA parameters.

Virgona (1992), a negative relationship between growth and $\Delta^{13}\text{C}$ may occur when constraints in photosynthetic capacity prevail over stomatal limitation. The $\Delta^{13}\text{C}$ value provides a

very good indication of the leaf intercellular concentration of CO_2 (C_i). High C_i is explained either by a low photosynthetic rate or, more commonly, by high stomatal conductance (Larcher, 2003). The higher $\Delta^{13}\text{C}$ in water-stressed plants may be explained by an increase in C_i as a consequence of the reduction in photosynthetic activity. This explanation is supported by the higher C_i found in the water-stressed ramets than in the well-watered ramets, although the difference was not significant. Increased $\Delta^{13}\text{C}$ in water-stressed plants may be related to a significant reduction in leaf nitrogen content (Guehl *et al.*, 1995; Sparks and Ehleringer, 1997; Retuerto *et al.*, 2000), as we observed in the water-stressed ramets. Most of the leaf nitrogen is bound in photosynthetic enzymes, and therefore the reduction in leaf nitrogen content reported in water-stressed ramets is consistent with the reduction in photosynthesis and growth observed in these plants. In short, the present results show that water stress reduced leaf nitrogen content and consequently photosynthetic activity and growth. However, $\Delta^{13}\text{C}$ increased because photosynthetic constraints prevailed over stomatal conductance in determining C_i . Similar results have been reported by Sparks and Ehleringer (1997), who found decreased leaf nitrogen and photosynthesis but increased $\Delta^{13}\text{C}$ values in woody species. In addition, Aranda *et al.* (2010) reported that, within some species, populations from more xeric sites were not those with the lowest $\Delta^{13}\text{C}$ values, suggesting that other species-specific traits, such as specific leaf area and nitrogen content, may modulate the isotopic signal.

The nitrogen form (ammonium or nitrate) on which the offspring ramets grew determined the nitrogen isotopic composition ($\delta^{15}\text{N}$) of their tissues. The $\delta^{15}\text{N}$ of offspring ramets growing in ammonium was significantly lower than that in offspring ramets growing in nitrate, for both connected and severed ramets. Similarly, other studies have found that an increase in ammonium uptake leads to a decrease in $\delta^{15}\text{N}$ (Evans, 2001; Kahmen *et al.*, 2008). Thus, our results support the use of the nitrogen isotopic signature ($\delta^{15}\text{N}$) as a useful tool for determining the nitrogen form used by plants. However, we found no evidence to support our second hypothesis. In contrast to our expectations, there was no preferential transport of N from ammonium or nitrate sources from parents to offspring ramets, to supply these ramets with the nitrogen

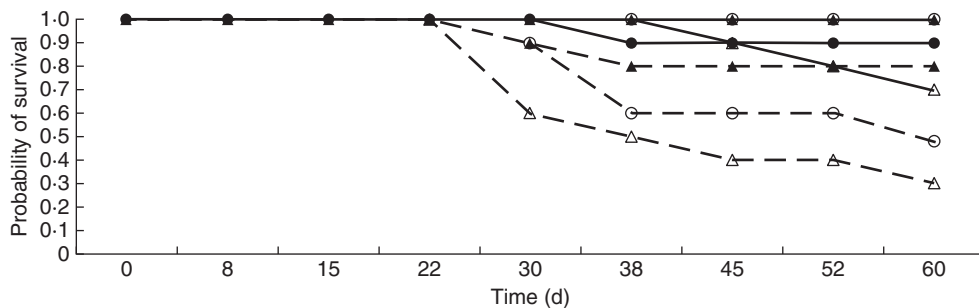


FIG. 7. Time course of the Kaplan–Meier unconditional survival probability for connected (solid lines) and severed (dashed lines) offspring ramets growing in the well-watered (closed symbols) and water-stressed (open symbols) treatments and receiving nitrate (circles) or ammonium (triangles) as the only form of nitrogen. See text for log rank (Mantel–Cox) test.

form in which they were deficient. Rather, our results (Fig. 4C, D) showed that connection, in water-stressed ramets, decreased $\delta^{15}\text{N}$ values, suggesting that offspring mainly received N from ammonium sources, whereas connection in well-watered ramets increased $\delta^{15}\text{N}$ values, which suggests that these ramets could be obtaining mainly nitrates from their parents. As a result, $\delta^{15}\text{N}$ values in connected water-stressed ramets were within the range of values found for plants grown in ammonium, whereas $\delta^{15}\text{N}$ values in connected well-watered ramets resembled the values in plants grown with nitrate as the only nitrogen form. However, $\delta^{15}\text{N}$ of severed offspring was similar regardless of the water availability conditions. These results represent a novel finding that provide compelling evidence of preferential transport of N from the ammonium source (parents) to water-stressed offspring, giving support for our third hypothesis. Since ammonium is toxic to plants and typically not found in the xylem, it is likely that, after uptake in the roots, the ammonium is incorporated immediately into glutamate, which is the main transport agent of reduced N in plants. One plausible reason for the preferential transport of ammonium from parents to water-stressed offspring is that water consumption in plants may be affected by the form of nitrogen that they receive (Guo *et al.*, 2007). Previous studies have shown that plants supplied with ammonium consume less water than those supplied with nitrate. Høgh-Jensen and Schjoerring (1997) found that the water-use efficiency of the clonal *Trifolium repens* was higher in plants supplied with ammonium than in those supplied with nitrate. Similar results have been reported for wheat (Yin and Raven, 1998). We interpret the preferential transport of N from the ammonium source as a mechanism for reducing water consumption in water-stressed ramets. Higher aquaporin activity in plants with ammonium nutrition than in plants with nitrate nutrition (Gao *et al.*, 2010) is consistent with our interpretation. This is a novel result that represents a potential benefit of clonal integration in terms of water consumption that has not been reported previously, and therefore this research extends the concept of physiological integration in clonal plants to include preferential transport of nutrients.

Previous studies have found both physiological and morphological compensatory responses of parents in response to demands from offspring ramets growing in unfavourable conditions. Experiments with *F. vesca* showed that photosynthetic efficiency in parent ramets increased in response to the assimilate demand made by drought-stressed offspring ramets (Roiloa and Retuerto, 2007). Similarly, parent ramets of *F. vesca* increased

the biomass allocated to roots as a non-local compensatory response to the copper-polluted soil in which the offspring ramets were placed (Roiloa and Retuerto, 2012). However, this is the first study suggesting preferential transport of a specific form of nitrogen in order to optimize water use by a member of the clone. The preferential transport of N from the ammonium source (parents) to water-stressed offspring ramets is consistent with three well-known facts: (1) ammonium uptake generally occurs at a higher rate than nitrate uptake for a wide diversity of species of different functional types (Lee and Stewart, 1978; Marty *et al.*, 2009; Mota *et al.*, 2011; Gherardi *et al.*, 2013; Lian *et al.*, 2012; Yang *et al.*, 2013); (2) a water potential gradient facilitates the movement of resources between connected ramets (Stuefer and Hutchings, 1994; Jónsdóttir and Watson, 1997); and (3) in clonal plants acropetal transport (from the parent to the offspring ramet) of resources exceeds basipetal transport (from offspring to the parent ramet) (Alpert and Mooney, 1986; Price and Hutchings, 1992; Alpert, 1996). Although it is clear that our results suggest a potential benefit of clonal integration in terms of water consumption for plants that are water-stressed, we should not necessarily expect an increase in biomass in plants growing in ammonium. In fact, previous studies have demonstrated that, compared with nitrate as an N source, ammonium results in small root and small leaf area, which may contribute to a low carbon gain and inhibition of growth (Guo *et al.*, 2007). Substantially higher losses of carbon through root and leaf respiration have been found with ammonium than with nitrate (Bloom *et al.*, 1992; Peuke and Jeschke, 1993; Cramer and Lewis, 1993).

Finally, the probable preferential transport of N from the ammonium source to the water-stressed ramets is also consistent with the unexpected higher $\Delta^{13}\text{C}$ values we found in these ramets. Previous studies have detected higher $\Delta^{13}\text{C}$ values in ammonium-supplied plants than in nitrate-supplied plants (Raven and Farquhar, 1990). Therefore, the increased $\Delta^{13}\text{C}$ in water-stressed offspring ramets could also be explained by the preferential transport of N from the ammonium source to the offspring ramets.

Conclusions

Summarizing, the present findings suggest preferential transport of N from the ammonium source to offspring ramets of *F. vesca* growing under water-stressed conditions. In the light of the lower water consumption due to ammonium use, we

interpret this result as a compensatory response in order to improve water use by the clone. This novel finding deepens and extends the concept of physiological integration in clonal plants to include preferential transport of nutrients. Our study contributes a novel perspective to the understanding of the complementary use of different forms of nitrogen by the different components of a clonal system. Integration improved survival of offspring ramets growing in water-stressed conditions, providing evidence of the importance of physiological integration in maintaining the presence of ramets in unfavourable conditions. We also demonstrate the benefit of integration, regardless of water availability or nitrogen form, in terms of photochemical activity and photosynthesis, which has scarcely been studied. Finally, this is the first study in which carbon and nitrogen isotopic composition has been used to detect physiological integration in clonal plants. Physiological integration enables clonal plants to buffer stressful conditions, and therefore this clonal trait should be considered as a key contribution to the success of clonal growth in plant communities.

ACKNOWLEDGEMENTS

We thank R. Bermúdez-Villanueva and X. Santiso for assistance in the greenhouse. We are grateful to M. Lema of SAI-UTIA (University of A Coruña) for carbon and nitrogen isotope analyses of leaves, to M. Santiso of the Department of Edaphology (University of Santiago de Compostela) for ammonium and nitrate soil content analyses, and Christine Francis for English correction. We are grateful to two anonymous referees and to the editor Ming Dong for their valuable comments on an earlier version of this paper.

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