

PHYSIOLOGICAL SCREENING OF RUDERAL WEED BIOMONITORS OF ATMOSPHERIC NITROGEN DEPOSITION

EVALUACIÓN FISIOLÓGICA DE MALEZAS RUDERALES COMO BIOMONITORES DE DEPÓSITO DE NITRÓGENO ATMOSFÉRICO

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Abstract

Background: Plants take up various species of reactive nitrogen and their different physiological responses to the increase of nitrogen availability can be useful in biomonitoring.

Questions: Does atmospheric nitrogen deposition affect the physiology of ruderal weeds? Which species are most responsive to nitrogen deposition?

Studied species: Eleven ruderal weeds.

Study site and dates: Morelia, Michoacán, Mexico. 2019.

Methods: Under scenarios of 10, 20, 40 and 80 kg N ha⁻¹ year⁻¹, we quantified plant responses of biomass production, nitrate reductase activity, chlorophyll content, photosynthetic efficiency, $\delta^{15}\text{N}$, nitrogen and carbon content.

Results: Total biomass production increased with the rate of nitrogen deposition for *Bidens pilosa*, *Chloris gayana*, *Lepidium virginicum*, and *Pennisetum setaceum*, as chlorophyll content in *B. pilosa*, *C. gayana*, and *L. virginicum*. In turn, the below- to above-ground biomass ratio decreased for *B. pilosa* and *C. gayana*, as photosynthetic efficiency in *C. gayana*, *L. virginicum*, and *Chloris pycnothrix*. Nitrate reductase activity was only affected in *L. virginicum*, *C. gayana*, and *T. officinale*.

With the exception of *C. pycnothrix*, the nitrogen content increased, while the carbon augmented in *C. gayana*, *C. pycnothrix*, and *P. setaceum*. The C/N ratio was reduced in *B. pilosa*, *C. gayana*, *Chloris virgata*, *P. setaceum*, and *T. officinale*. The $\delta^{15}\text{N}$ was increased in *B. pilosa*, *C. gayana*, *C. virgata* and *P. setaceum*.

Conclusions: *Bidens pilosa*, *C. gayana*, *L. virginicum*, and *P. setaceum* were the species with more affected variables to nitrogen deposition, which could be useful in the biomonitoring.

Keywords: Atmospheric pollution, biomass, chlorophyll, nitrate reductase, plant nutrition, stable isotopes

Resumen

Antecedentes: Las plantas asimilan formas reactivas de nitrógeno y sus respuestas fisiológicas al incremento en la disponibilidad de nitrógeno pueden ser de utilidad en el biomonitoreo.

Preguntas: ¿Cómo responde la fisiología de malezas ruderales al depósito de nitrógeno atmosférico? ¿Cuáles especies son sensibles al depósito de nitrógeno?

Especies de estudio: Once malezas ruderales

Sitio y años de estudio: Morelia, Michoacán, México. 2019.

Métodos: Se cuantificó la producción de biomasa, actividad de la enzima nitrato reductasa, contenido de clorofila, eficiencia fotosintética, $\delta^{15}\text{N}$, contenidos de N y C bajo escenarios de 10, 20, 40 y 80 kg N ha⁻¹ año⁻¹.

Resultados: La biomasa total incrementó con el depósito de nitrógeno en *Bidens pilosa*, *Chloris gayana*, *Lepidium virginicum* y *Pennisetum setaceum*, al igual que la clorofila en *B. pilosa*, *C. gayana* y *L. virginicum*. La relación biomasa subterránea/aérea se redujo en *B. pilosa* y *C. gayana*, así como la eficiencia fotosintética en *C. gayana*, *L. virginicum* y *Chloris pycnothrix*. La actividad de la enzima nitrato reductasa se afectó en *L. virginicum*, *C. gayana* y *Taraxacum officinale*.

A excepción de *C. pycnothrix*, el contenido de nitrógeno aumentó, mientras el carbono incrementó en *C. gayana*, *C. pycnothrix* y *P. setaceum*. La relación C/N se redujo en *B. pilosa*, *C. gayana*, *Chloris virgata*, *P. setaceum* y *T. officinale*. El $\delta^{15}\text{N}$ se incrementó en *B. pilosa*, *C. gayana*, *C. virgata* y *P. setaceum*.

Conclusiones: *Bidens pilosa*, *C. gayana*, *L. virginicum* y *P. setaceum*, las especies con más variables afectadas por el depósito de nitrógeno, podrían ser útiles en el biomonitoreo.

Palabras clave: Biomasa, clorofila, contaminación atmosférica, isótopos estables, nitrato reductasa, nutrición vegetal.

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The amount of reactive nitrogen (Nr) species available on the planet has increased as a result of human activities, such as fossil fuel combustion and the utilization of nitrogenous fertilizers (Galloway *et al.* 2003, 2004). Indeed, the release of Nr to the environment increased from 33 Tg N year⁻¹ in 1860 to 156 Tg N year⁻¹ in 1990, and it is anticipated that this rate will double by 2050 (Galloway *et al.* 2004). Such alterations to the nitrogen biogeochemical cycle are a major component of global environmental change and a principal threat to the planet's biodiversity (Sala *et al.* 2000, Rockström *et al.* 2009). In particular, given that many plants evolved in environments with limited nitrogen availability (Lee & Caporn 1998), the increase of this macronutrient has enabled those species capable of attaining rapid growth rates and of tolerating the ensuing toxic ion buildup to become better ecological competitors (Bobbink *et al.* 1998, 2010, Stevens *et al.* 2004, Farrer *et al.* 2013). In addition, the deposition of reactive nitrogen species is related to eutrophication, soil acidity, and cation leaching (DeHayes *et al.* 1999, Kronzucker *et al.* 2001, Britto & Kronzucker 2002, Galloway *et al.* 2003, Conklin 2005, Gruber & Galloway 2008, Bobbink *et al.* 2010, Persson *et al.* 2010, Tian *et al.* 2016). However, nitrogen deposition is not only linked with ecosystem and environmental problems, as it also has noxious effects on human health, such as intoxication by a high concentration of NO₃⁻ and NO₂ in drinking water that affects the early developmental stages of our development (Carpenter *et al.* 1998, WHO 2003). Reactive nitrogen is thus an important component of pollution, which leads to 16 % of premature deaths worldwide (Landrigan *et al.* 2018, Yeo *et al.* 2019).

Given the multiple noxious effects of environmental pollution, it becomes necessary, and in some instances required by law, to monitor and control the levels of pollutants that are released to the environment. However, for the case of atmospheric pollution, the deployment, operation, and maintenance of monitoring systems is complicated and can be cost-prohibitive for local governments (SEMARNAT 2012, Díaz-Álvarez *et al.* 2018). In this respect, the utilization of naturally occurring biomonitors has been proposed as an alternative for localities where air quality monitoring systems are lacking (Arróniz-Crespo *et al.* 2012, Felix *et al.* 2016, Díaz-Álvarez *et al.* 2018). Indeed, various ecophysiological traits can be utilized to characterize nitrogen deposition in regions of interest, including an increase in tissue nitrogen content and a subsequent imbalance in the C/N ratio, as well as changes in the activity of enzymes related to the nitrogen metabolism, such as nitrate reductase, and the rate of ¹⁵N isoto-

pic discrimination (Sutton *et al.* 2004, Arróniz-Crespo *et al.* 2008, Felix *et al.* 2016, Díaz-Álvarez & de la Barrera 2018, Díaz-Álvarez *et al.* 2015, 2019, 2020). In general, an increase in nitrogen availability from atmospheric deposition can improve biomass accumulation and reduce the allocation to below-ground tissues relative to aerial tissues (Li *et al.* 2015). Additional nitrogen can be stored in the inorganic form within organelles or as Rubisco, leading to an increase of chlorophyll content and the plant's photosynthetic capacity (Arróniz-Crespo *et al.* 2008, Jin *et al.* 2015, Tegeder & Masclaux-Daubresse 2018). However, when nitrogen reaches a species-specific threshold, plants manifest symptoms of stress, such as changes in pH, membrane function and integrity, and energy deficiencies, which can be reflected in the maximum quantum yield of photosystem II (F_v/F_m) and the chlorophyll *a/b* ratio (Kronzucker *et al.* 2001, Britto & Kronzucker 2002, Arróniz-Crespo *et al.* 2008).

Plant species that rely exclusively or predominantly on atmospheric sources of mineral nutrition are particularly suited for biomonitoring. Such is the case for the bromeliad *Tillandsia recurvata* L. that can track dry nitrogen deposition, especially NO_x and particulate matter (Díaz-Álvarez & de la Barrera 2018), and various bryophytes that, in turn, are useful biomonitors of wet nitrogen deposition (Arróniz-Crespo *et al.* 2008, Díaz-Álvarez & de la Barrera 2018, Díaz-Álvarez *et al.* 2019, 2020). However, these so called "atmospheric biomonitors" cannot be utilized in all localities for various reasons, including that the abundance of epiphytes decreases away from the humid tropics (Zotz & Bader 2009), that mosses require high humidity environments to maintain physiological function (Glime 2017a), and that the prevalent pollution can be too high for certain species, as it occurs for *T. recurvata* in certain regions of Mexico City (Díaz-Álvarez & de la Barrera 2018).

Ruderal weeds are a group of plants that successfully establish in high-pollution environments, such as those found in cities, at least seasonally. These plants have also been utilized as pollution biomonitors, despite that their root system is anchored to the ground, having access to existing nutrients from sources different from atmospheric deposition (Norra *et al.* 2005, Wang & Pataki 2010).

Based on the hypothesis that plant physiological attributes will respond to an increase in the availability of reactive inorganic nitrogen species, we conducted a dose-response greenhouse experiment to screen some ruderal weeds as potential biomonitors of nitrogen deposition based on their physiological responses to nitrogen deposi-

tion. We expect that higher nitrogen availability will lead to increased biomass accumulation, tissue nitrogen and chlorophyll content, as well as higher rates of isotopic discrimination of ^{15}N , due to the fertilization effect. Also, the BS/BA and C/N ratios, and the nitrate reductase enzyme activity are expected to decrease. In turn, as responses to stress due to an increase in the availability of N and its toxicity, the chlorophyll a/b ratio, and the F_v/F_m are expected to decrease.

Material and methods

Experimental setup. Seeds of eleven ruderal weeds that are common in the city of Morelia, Michoacán, México (Table 1) were collected in October 2017 and 2018. The seeds were placed in paper envelopes (8.8×16.4 cm) and stored in the laboratory (in the dark, air temperature of 23 °C, and relative humidity of 40 %) until they were utilized in February 2019.

The experiment was conducted in a greenhouse at the Escuela Nacional de Estudios Superiores, Unidad Morelia, Universidad Nacional Autónoma de México, Morelia, Michoacán, Mexico, where the plants were exposed to an air temperature averaging 17 °C throughout the experiment (range of 2 to 36 °C), a relative humidity of 59 % (5-97 %), and a daily photosynthetic photon flux (wavelengths of 400 to 700 nm) of $18.6 \text{ mol m}^{-2} \text{ day}^{-1}$. Plants were exposed to four treatments that simulated nitrogen deposition rates of 10, 20, 40, and 80 Kg N $\text{ha}^{-1} \text{ year}^{-1}$ over 120 days after sowing. Each treatment had six replicates per species. The experimental units of all species were

randomly distributed on a greenhouse bench (dimensions of 15×1.5 m) to avoid blocking effects on individual species. For each experimental unit, at least three seeds were sown in plastic pots (volume of 1.5 liters) containing agrolite. Following germination, the most vigorous individual was kept for the experiment, while the remaining seedlings were removed. Two sets of each experimental unit were prepared, considering that part of the experiment involved destructive sampling of plant material.

Nitrogen deposition. Because ruderal weeds are predominantly active during the rainy season, the experiment evaluated responses to wet deposition simulated with aqueous solutions of NH_4NO_3 . In particular, the total amount of nitrogen that would deposit under rates of 10, 20, 40, and 80 Kg N $\text{ha}^{-1} \text{ year}^{-1}$, were administered over four months, the typical duration of the rainy season in the study region (Morelia has a C(w) climate, i.e., temperate, subhumid, with summer rains, INEGI 2017). Taking into consideration an opening area of $9.5 \times 10^{-3} \text{ m}^2$, each pot was watered daily, during 120 days, with 140 ml of 0.0161, 0.032, 0.64, or 0.128 mM NH_4NO_3 . Additionally, the plants received weekly irrigations with 140 ml of a modified 0.1 Hoagland solution lacking nitrogen, in order to avoid nutrient deficiencies (Nobel & de la Barrera 2002). At the end of the 120-days, the biological material was harvested and analyzed.

Biomass. The plants were harvested at the end of the experiment and dried at 45 °C in a gravity convection oven until reaching constant weight. Below-ground (BS) and

Table 1. Ruderal weeds common in Morelia, Michoacán, México, whose biomonitoring potential was evaluated.

Species	Family	Class	Origin ^a
<i>Amaranthus hybridus</i> L.	Amaranthaceae	Magnoliopsida	Native
<i>Bidens pilosa</i> L.	Asteraceae	Magnoliopsida	Native
<i>Chloris gayana</i> Kunth	Poaceae	Liliopsida	Exotic
<i>Chloris pycnothrix</i> Trin.	Poaceae	Liliopsida	Exotic
<i>Chloris virgata</i> Sw.	Poaceae	Liliopsida	Exotic
<i>Lepidium virginicum</i> L.	Brassicaceae	Magnoliopsida	Native
<i>Melinis repens</i> L.	Poaceae	Liliopsida	Exotic
<i>Pennisetum ciliare</i> (L.) Link	Poaceae	Liliopsida	Exotic
<i>Pennisetum setaceum</i> (Forssk.) Chiov.	Poaceae	Liliopsida	Exotic
<i>Sporobolus indicus</i> (L.) R. Br.	Poaceae	Liliopsida	Native
<i>Taraxacum officinale</i> (L.) Weber ex F.H.Wigg.	Asteraceae	Magnoliopsida	Exotic

^a Rzedowski & Rzedowski 2005, Vibrans 2021.

above-ground (BA) dry mass were determined separately, in order to calculate the BS/BA ratio, as well as the total biomass accumulation for each individual.

Elemental and isotopic analyses. Plant material was harvested, dried to constant weight in the gravity convection oven at 60 °C, and ground to a fine powder prior to submission to the Stable Isotope Facility, University of Wyoming for elemental and isotopic analyses that were conducted with a Costech 4010 elemental analyzer (Costech Analytical Inc., Valencia, California, USA) attached to a continuous flow isotope ratio mass spectrometer (Finnigan Delta Plus XP, Thermo Electron Corp, Waltham, Massachusetts, USA). The analytical precision was 0.4 ± 0.03 (SD) for the $\delta^{15}\text{N}$.

Chlorophyll. Chlorophyll content was determined colorimetrically (Lichtenthaler 1987). Freshly harvested leaf samples were macerated with cold acetone (80 % v/v in distilled water), and brought to a final volume of 3.0 ml. The absorbance of filtered aliquots was measured with an EZ 301 Spectrometer (Perkin Elmer, Waltham, Massachusetts, USA).

The maximum quantum yield of photosystem II (F_v/F_m ; Maxwell & Johnson 2000), was measured with a FluorPen FP 100 hand-held fluorometer (Photon Systems Instruments, Drasov, Czech Republic), for plants that had been dark acclimated for 20 min by covering the entire pot with a brown paper bag that lined with aluminum foil (DeEll & Toivonen 2011).

Nitrate reductase activity (NR). The enzymatic activity of the nitrate reductase was quantified by colorimetry, based on the Greis-Ilosuay reaction (Díaz-Álvarez *et al.* 2019). Leaf samples were incubated in a 3 mM KNO_3 solution during 12 hr, followed by the addition of 5 ml of a potassium buffer solution (50 mM KH_2PO_4 , 100 mM KNO_3 , 100 mM potassium acetate, and 1.5 % v/v propanol-1-ol). After 1-2 min, the vials were emptied and incubated in an orbital shaker at 30 °C during 30 min, before reading absorbance at 540 nm with the EZ 301 Spectrometer.

Data analyses. Plant survival throughout the experiment was analyzed with Friedman repeated measures ANOVAs, followed by post hoc Tukey tests ($P \leq 0.05$). For the rest of the parameters, *i.e.*, biomass production, BS/BA, NR activity, chlorophyll content, chlorophyll *a/b* ratio, F_v/F_m , $\delta^{15}\text{N}$, C/N ratio, N, and C content, plant responses were analyzed one-way ANOVAs followed by post hoc Tukey

or Student's *t* tests ($P \leq 0.05$). When the normality and variance homogeneity requirements were not fulfilled, data were analyzed with Kruskal-Wallis tests followed by Tukey or Dunn tests ($P \leq 0.05$). Data are shown as mean ± 1 S.E. ($n = 6$). Statistical analyses were conducted with SigmaStat 3.5 (Systat Software Inc., San Jose, California).

Results

Survival for the weeds considered in the present work had different responses to the experimental treatments over 120 days after sowing (Figure 1; Supplementary material). In particular, the lowest dose of 10 kg N ha⁻¹ year⁻¹ led to the highest survival for *Pennisetum ciliare* (L.) Link (Figure 1C) but to the lowest survival for *Chloris pycnothrix* Trin. (Figure 1G), and *Melinis repens* (Willd.) Zizka (Figure 1B). In turn, the survival for *Amaranthus hybridus* L. (Figure 1A), *Lepidium virginicum* L. (Figure 1I), and *Pennisetum setaceum* (Forssk.) Chiov. (Figure 1J), was the highest under some of the intermediate scenarios of nitrogen deposition, and the lowest survival for *Sporobolus indicus* (L.) R. Br. (Figure 1D). The highest dose of 80 Kg N ha⁻¹ year⁻¹ led to the lowest survival for *P. setaceum* (Figure 1J), but to the highest survival for *Chloris virgata* Sw. (Figure 1H) and *Taraxacum officinale* (L.) Weber ex F.H.Wigg (Figure 1K). Finally, the survival for *Bidens pilosa* L. (Figure 1E; $P = 0.029$) and *Chloris gayana* Kunth (Figure 1F) did not respond to the experimental treatments and it was very low throughout the experiment.

Given that only seven out of the eleven species that were evaluated had a final survival of at least 33 % after 120 days, *i.e.*, *Bidens pilosa* (Figure 1E), *Chloris gayana* (Figure 1F), *C. pycnothrix* (Figure 1G), *C. virgata* (Figure 1H), *Lepidium virginicum* (Figure 1I), *Pennisetum setaceum* (Figure 1J) and *Taraxacum officinale* (Figure 1K), the remaining four species were excluded from further physiological screening.

Similar to the case for survival, the sensitivity of the various physiological parameters evaluated responded differently for each species under the different nitrogen doses (Figure 2; Supplementary material). Biomass accumulation tended to increase under the higher nitrogen doses for *Bidens pilosa* (Figure 2A), *Chloris gayana* (Figure 2B), *Lepidium virginicum* (Figure 2E), and *Pennisetum setaceum* (Figure 2F), with an ensuing decrease in the BS/BA ratio for the former two. In addition, the tissue carbon content increased with the nitrogen dose for *C. gayana* and *P. setaceum*, but it decreased for *C. pycnothrix* (Figure 2C).

In general, the nitrogen content of the seven weeds

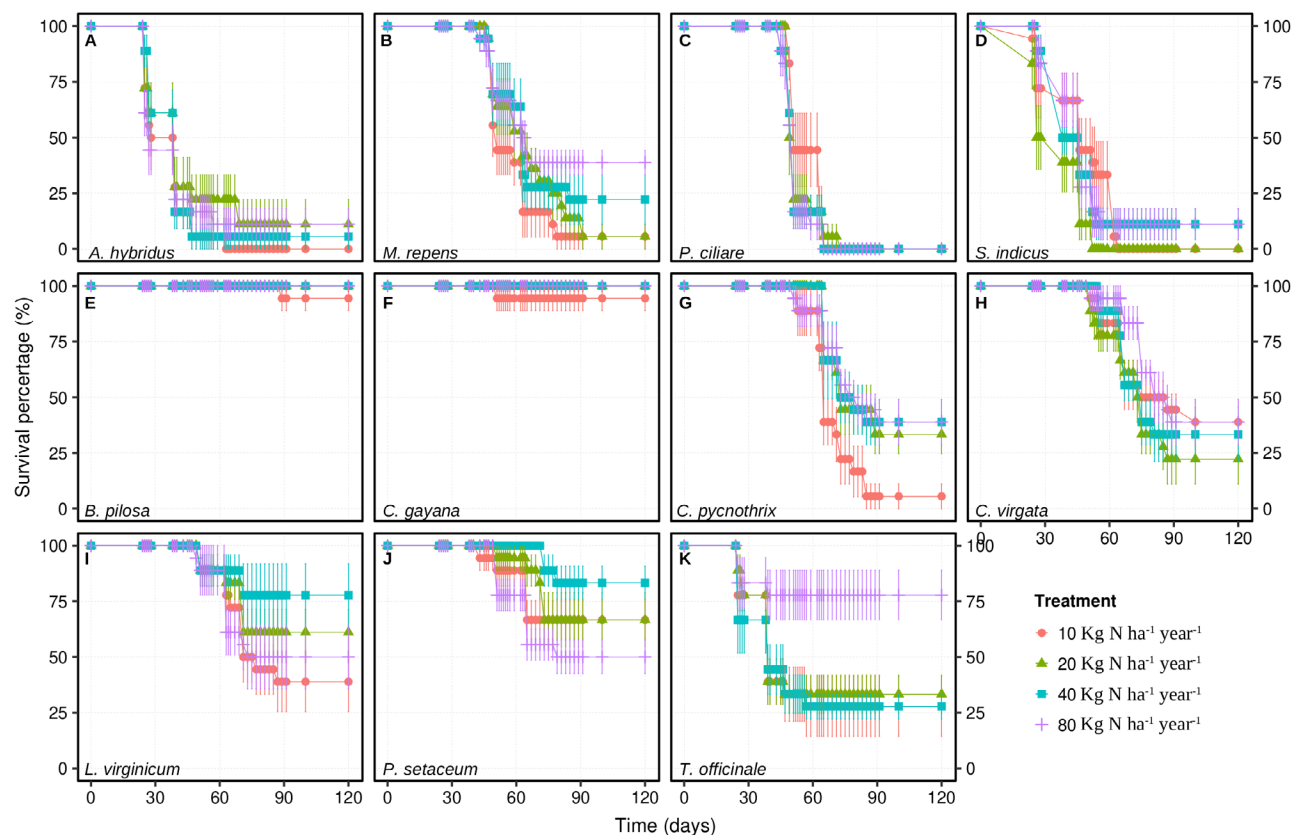


Figure 1. Survival over 120 days for 11 potential ruderal weed biomonitors under experimental nitrogen deposition. Data are shown as mean \pm 1 standard error ($n = 6$). Please refer to the Supplementary material for data analyses.

tended to increase with the nitrogen dose, except for *C. pycnothrix*, which did not respond to the experimental treatment, and for *L. virginicum*, for which the tissue nitrogen content was maximal under the intermediate dose of 40 kg ha⁻¹ year⁻¹. The higher tissue nitrogen content was followed by a decrease in the C/N ratio for *B. pilosa*, *C. gayana*, *C. virgata* (Figure 2D), *P. setaceum*, and *T. officinale* (Figure 2G). In turn, the $\delta^{15}\text{N}$ values tended to become less negative under the higher nitrogen doses for *B. pilosa*, *C. gayana*, *C. virgata*, and *P. setaceum*, but remained unaffected for the other three species. The activity of the nitrate reductase tended to decrease with the nitrogen dose for *C. gayana* and *T. officinale*, it reached its maximum under 40 kg ha⁻¹ year⁻¹ for *L. virginicum* and remained unaffected for the other species.

The total chlorophyll content tended to increase with the nitrogen dose for *B. pilosa*, *C. gayana*, and *L. virginicum*. The chlorophyll a/b ratio decreased for *C. gayana* under the nitrogen doses above 10 kg N ha⁻¹ year⁻¹, and for *L. virginicum* growing under 80 kg N ha⁻¹ year⁻¹. Finally, F_v/F_m for dark-adapted leaves did not respond to ni-

trogen deposition, except for *C. pycnothrix*, for which it decreased under 80 kg N ha⁻¹ year⁻¹.

Discussion

While most of the species screened here responded to the experimental nitrogen deposition, a generalized pattern was not observed, neither for the sensitivity of the physiological parameters that were measured nor by groups of species. For instance, one could have expected that exotic species, especially those reported as invasive would have fared better than the natives at higher nitrogen doses (van der Maarel 2005, Perry et al. 2010). However, this was not the case, as the survival of some of the weeds, i.e., *Bidens pilosa* and *Chloris gayana*, were insensitive to nitrogen addition, others, i.e., *Chloris pycnothrix* and *Taraxacum officinale*, had a decreased mortality with increasing fertilization, while *Pennisetum ciliare*, a very noxious invasive weed, succumbed under all the treatments. Even *C. pycnothrix*, an alien species, showed stress effects due high nitrogen deposition (a reduced F_v/F_m) than some of the na-

tive species. Such an idiosyncratic response to nitrogen deposition is prevalent in nature: while the survival of species such as the grass *Deschampsia flexuosa* (L.) Trin., the shrub *Calluna vulgaris* (L.) Hull, and the trees *Fraxinus americana* L., *Malus coronaria* L., and *Schima superba* Gardner & Champ. are insensitive to nitrogen addition (van den Berg *et al.* 2005, McWhirter & Henry 2014, Han *et al.* 2019), plant mortality decreases with nitrogen availability for *Elaeagnus umbellata* Thunb., *Erodium oxyrhinchum* M. Bieb., and *Robinia pseudocacia* L. (McWhirter & Henry 2014, Horn *et al.* 2018, Chen *et al.* 2019a), but it increases for species such as *Succisa pratensis* L., *Antennaria dioica* L., *Pinus massoniana* D. Don, *Pouteria torta* (Mart.) Radlk., and 39 species of trees (van den Berg *et al.* 2005, Cárate-Tandalla *et al.* 2015, Horn *et al.* 2018, Han *et al.* 2019). Such an increased mortality in nitrogen-rich environments has been attributed to a low tolerance to NH_4^+ , whose accumulation leads to acidification (Britto & Kronzucker 2002, van den Berg *et al.* 2005), as well as to energy deficits resulting from the cost of extruding excess NH_4^+ out of the cell (Kronzucker *et al.* 2001, Britto & Kronzucker 2002, van den Berg *et al.* 2005). The responses of individual species lead to changes in their distribution, which combined alter the composition of plant communities (Gotelli & Ellison 2002, Horn *et al.* 2018).

Some of the responses observed here, however, could simply be attributed to each species' habitat preference. For instance, *Melinis repens* and *Taraxacum officinale* have been found to establish successfully in urban environments, as long as the ground has sufficient amounts of litter or nurse plants are available (Cavieres *et al.* 2005, David & Menges 2011). Also, a high mortality is inherent of species that produce a large number of propagules with a low investment of maternal resources (Ricklefs 2009), especially considering the high vulnerability of plants during early developmental stages (de la Barrera *et al.* 2009). This could be the case, for example, of *Pennisetum ciliare*, whose mortality reached 100 % in the present study, but which becomes insensitive to nitrogen fertilization once it has become established (Lyons *et al.* 2013).

As expected, a higher nitrogen availability increased biomass accumulation for some of the weeds, i.e., *Bidens pilosa*, *Chloris gayana*, *Lepidium virginicum*, and *Pennisetum setaceum*, a response that has also been documented for *Agropyron cristatum* (L.) Gaertn., *Anthoxanthum odoratum* L., *Avena fatua* L., *Centaurea stoebe* L., *Hordeum murinum* L., *Lolium perenne* L., *Medicago lupulina* L., *Plantago lanceolata* L., *Poa annua* L., *Prunella vulgaris* L., *Stipa pulchra* Hitchc., and *Trifolium repens* L. (Jiang

et al. 2005, Tian *et al.* 2012, Stevens & Gowing 2014, Peng *et al.* 2016, Tulloss & Cadenasso 2016, Shen *et al.* 2019). Such an improvement of primary productivity in response to fertilization is common when plants develop in nutrient-limited soils (Azcón-Bieto & Talón 2008, van der Valk 2009, Taiz *et al.* 2014). As additional nitrogen becomes available, biomass accumulation can increase linearly until a threshold is reached, either by a saturation of the response, an intrinsic limitation of the plant, the intracellular buildup of toxic ions, or by a co-limitation of other nutrients (Azcón-Bieto & Talón 2008, Taiz *et al.* 2014, J. Mao *et al.* 2018a). This was probably the case for *Chloris pycnothrix*, *C. virgata*, and *Taraxacum officinale*, whose dry mass accumulation did not respond to nitrogen fertilization. Insensitivity of growth to fertilization has also been documented for species such as *Amaranthus spinosus* L., *Elaeagnus umbellata*, *Elymus caput-medusae* L., *Eremopyrum orientale* (L.) Jaub. & Spach, *Fraxinus americana*, *Malus coronaria*, *Plantago virginica* L., *Rhus typhina* L., and *Schima superba* (Jiang *et al.* 2005, McWhirter & Henry 2014, Tulloss & Cadenasso 2016, Chen *et al.* 2019b, Han *et al.* 2019).

Resource allocation to belowground biomass that increases the BS/BA ratio is also a response of plants that grow in nutrient-poor soils, as a higher root surface area improves the ability to take up nutrients (Litton *et al.* 2003, Taiz *et al.* 2014). In turn, a higher nitrogen availability usually leads to a reduction of BS/BA, a response that has been observed in species such as *Nepeta micrantha* Bunge, *Oriza sativa* L., and that is common in forest species (Li *et al.* 2015, Mao *et al.* 2018b, Chen *et al.* 2019b, Wang *et al.* 2019). Despite that a reduction in BS/BA was only significant for two of the weeds, the remaining five displayed an apparent trend in the same direction, similar to what occurs for *Amaranthus spinosus*, *Eremopyrum orientale*, *Lolium perenne*, *Medicago lupulina*, *Poa annua*, *Prunella vulgaris*, and *Trifolium repens* (Jiang *et al.* 2005, Stevens & Gowing 2014, Chen *et al.* 2019a). However, a lack of response of BS/BA can be attributed to limitation of other soil nutrients, such that an investment in root tissue still improves soil exploration, potentially conferring a better competitive capacity (Tulloss & Cadenasso 2016).

The nitrate reductase enzyme reduces the oxidation level of NO_3^- to NO_2^- , catalyze one of the early steps in the nitrogen assimilation (Azcón-Bieto & Talón 2008, Tegeder & Masclaux-Daubresse 2018). This enzyme was affected by the nitrogen deposition in *Chloris gayana* and *Taraxacum officinale*, where the increase of nitrogen deposition rate reduced their activity, as also we can see in

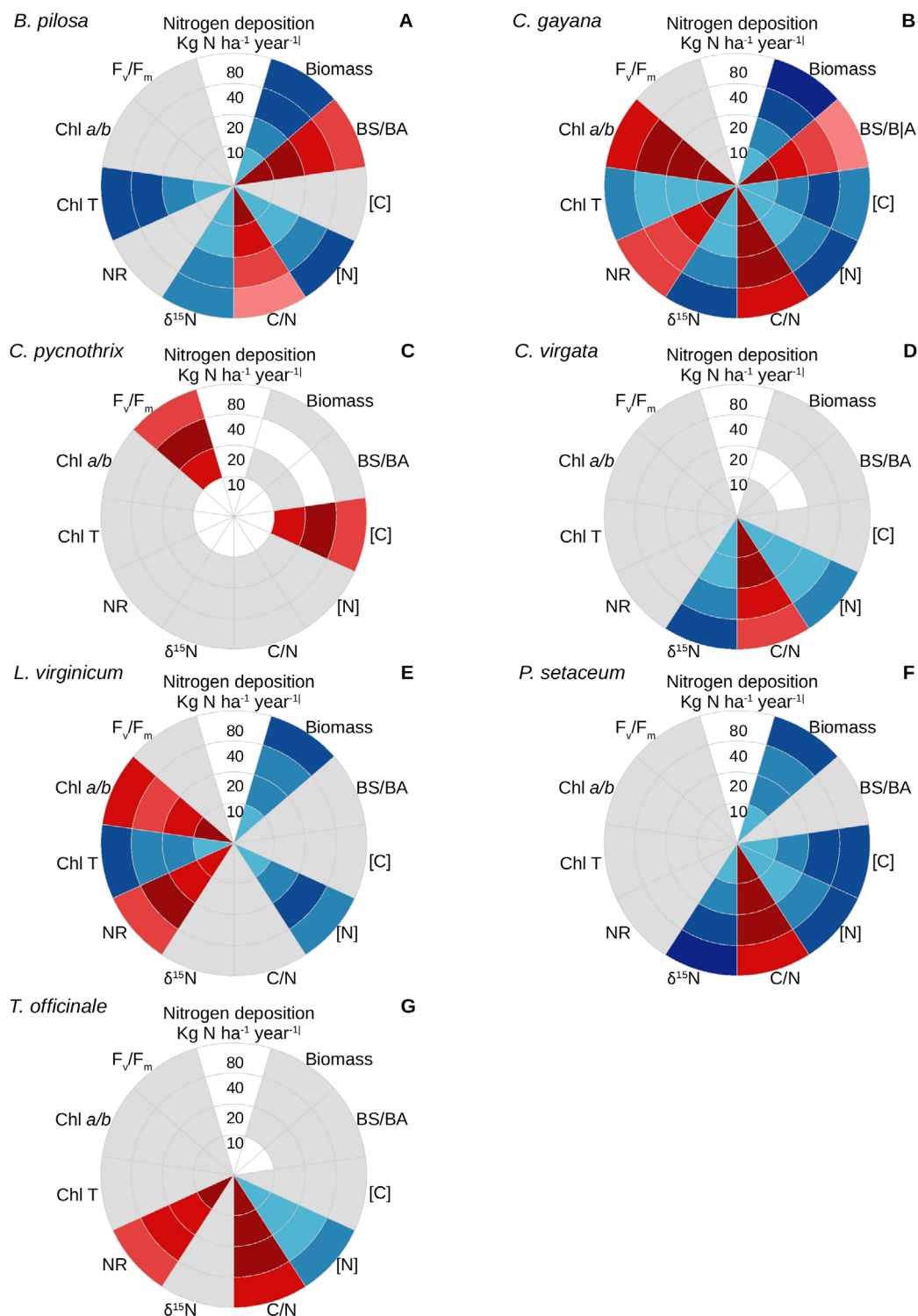


Figure 2. Physiological effects of experimental nitrogen deposition on the ruderal weeds (A) *Bidens pilosa*, (B) *Chloris gayana*, (C) *C. pycnorrhix*, (D) *C. virgata*, (E) *Lepidium virginicum*, (F) *Pennisetum setaceum*, and (G) *Taraxacum officinale*. For each species, the nitrogen dose increases outwards. Blue indicates that a given parameter had a direct response to the dose utilized, red indicates an inverse response, and grey indicates a lack of response to the different nitrogen doses. For each parameter, different color intensities indicate statistical differences in the magnitude of the response ($P < 0.05$). Please refer to the Supplementary material for the specific responses and data analysis of each parameter.

Acer saccharum Marshall and the bryophytes *Braunia secunda* (Hook.) Bruch & Schimp., *Leptodontium pungens* (Mitt.) Kindb., *Racomitrium lanuginosum* (Hedw.) Brid., and *Rhytidiadelphus squarrosus* (Hedw.) Warnst. (Pearce & van der Wal 2002, Arróniz-Crespo et al. 2008, Tang et al. 2012, Díaz-Álvarez et al. 2019). This reduction in the NR activity, under ascending scenarios of nitrogen deposition, is due to the increase of reduced nitrogen compounds in the tissues that inhibit their synthesis and activity (Downs et al. 1993, Arróniz-Crespo et al. 2008, Coelho & Romão 2015, Glime 2017b). In bryophytes, the decrease in activity is attributed to the available NH_4^+ satisfying the nitrogen demand, while in vascular plants it is attributed to intrinsic properties of species as growth rate and state of development (Downs et al. 1993, Arróniz-Crespo et al. 2008, Tang et al. 2012, Glime 2017b). Species that kept their NR activity constant under the scenarios of nitrogen deposition as *Bidens pilosa*, *Chloris pycnothrix*, *Chloris virgata* and *Pennisetum setaceum*, perhaps the NH_4^+ amount, that contributed the NH_4NO_3 with the treatments of nitrogen deposition, plus the NO_3^- reduced were enough to satisfy the nitrogen demand keeping the NR enzyme activity constant. The null effect of the nitrogen deposition in the NR enzyme has also been seen in *Acer rubrum* L., *Ardisia quinquegona* Blume, *Betula alleghaniensis* Britton, *Blastus cochinchinensis* Lour., *Fagus grandifolia* Ehrh., *Pinus strobus* L., *P. rigida* Mill., *Tillandsia recurvata* L., and the bryophytes *Pleurochaete squarrosa* (Brid.) Lindb. and *Pseudoscleropodium purum* (Hedw.) M. Fleisch. (Downs et al. 1993, Pearce & van der Wal 2002, Pearce et al. 2003, Arróniz-Crespo et al. 2008, Tang et al. 2012, Ochoa-Hueso & Manrique 2013, Liu et al. 2018b, Díaz-Álvarez et al. 2020).

The higher nitrogen content measured under higher doses for six of the weeds has also been reported for the mosses *Pseudoscleropodium purum* and *Rhytidium rugosum* (Hedw.) Kindb., and for the vascular plants *Calluna vulgaris* L., *Eucalyptus urophylla* S. T. Blake \times *E. grandis* Hill ex Maiden, *Laelia speciosa* (Kunth) Schltr., *Oxytropis kansuensis* Bunge, and *Pinus resinosa* L. (Throop & Lerdau 2004, Arróniz-Crespo et al. 2008, Bobbink et al. 2010, Du et al. 2014, 2015, Díaz-Álvarez et al. 2015, Lü et al. 2016). The nitrogen level in plant tissues is driven by vacuolar accumulation of reduced nitrogen species and hydrosoluble proteins, such as Rubisco (Zhang et al. 2016, Tegeder & Masclaux-Daubresse 2018). Nitrogen fertilization can also increase the PEPcarboxylase activity for C_4 and CAM species, leading to a concurrent increase of tissue carbon content, as it was observed here for

Chloris gayana, *C. pycnothrix*, and *Pennisetum setaceum* (Jin et al. 2015, Flexas et al. 2016, Tegeder & Masclaux-Daubresse 2018, Zhou et al. 2020).

The decrease in the C/N ratio in response to nitrogen fertilization that we found for *Bidens pilosa*, *Chloris gayana*, *Chloris virgata*, *Taraxacum officinale*, and *Pennisetum setaceum*, which also occurs for *Schizolobium amazonicum* Ducke, *Zea mays* L., as well as for various species in the *Cleistogenes* and *Stipa* genera, is a direct result of the nitrogen buildup described above (Chen et al. 2009, Luo et al. 2017, Vieira et al. 2018). In turn, the fact that the C/N ratio did not change for *Lepidium virginicum* and *Chloris pycnothrix* appears to be a consequence of the development of new plant tissue, which has also been documented for *Betula pendula* Roth, *Agrostis capillaris* L., and *Galium saxatile* L. (Stevens et al. 2011, Harmens et al. 2017).

The increased $\delta^{15}\text{N}$ values in response to higher nitrogen deposition rates such as those observed for *Bidens pilosa*, *Chloris gayana*, *Chloris virgata*, and *Pennisetum setaceum*, are opposite to those reported for species such as *Laelia speciosa* and *Pinus massonia* Lamb., whose leaves become increasingly impoverished in ^{15}N under higher nitrogen availability (Jiang & Zhang 2009, Díaz-Álvarez et al. 2015). An isotopic impoverishment of plant tissues indicates an enhanced discrimination of ^{15}N under an abundance of nitrogen resulting from increased enzyme-mediated processes (Yoneyama et al. 1991, Santiago et al. 2005, Xiao et al. 2011). In the present work, however, the negative $\delta^{15}\text{N}$ values found under the lower nitrogen doses can be an indication of high enzymatic activity, as it was the case, for instance, for *Chloris gayana* whose lowest $\delta^{15}\text{N}$ values occurred concurrently with its highest nitrate reductase activity. Thus, our results could be an indicator of enzyme saturation that prevented further nitrogen uptake. In addition, the observed isotopic enrichment may also be reflecting an increased loss of excess nitrogen in the plant by NH_4^+ volatilization or NO_3^- leaching (Höglberg & Johannisson 1993, Dijkstra et al. 2003, Jiang & Zhang 2009, Ma et al. 2012).

The higher chlorophyll content found for *Bidens pilosa*, *Lepidium virginicum*, and *Chloris gayana*, in response to increasing nitrogen availability is common in nature, and has been observed for species such as *Ardisia quinquegona*, *Camellia japonica* L., *Fraxinus mandshurica* Rupr., *Lindera aggregata* (Sims) Kosterm., *Pleurochaete squarrosa*, and *Populus cathayana* Rehder. (Arróniz-Crespo et al. 2008, Wang et al. 2012, Yuan et al. 2017, C. Liu et al. 2018a). However, in other cases the chlorophyll

content is insensitive to the prevalent nitrogen availability, as it occurs for species like *Calamagrostis angustifolia* Kom., *Quercus acutissima* Carruth., *Blastus cochinchinensis*, *Cryptocarya chinensis* (Hance) Hemsl., *C. concinna* Hance, *Randia canthioides* Champ. ex Benth., and *Populus deltoides* W. Bartram ex Marshall, or as found here for as found for *Chloris pycnothrix*, *C. virgata*, *Pennisetum setaceum*, and *Taraxacum officinale* (Dou *et al.* 2009, Li *et al.* 2018, Liu *et al.* 2018b, Liu *et al.* 2018c, Mao *et al.* 2018b, Xu *et al.* 2018). For these cases, chlorophyll content may be driven by other environmental factors, especially the prevalent photon flux density, which was relatively high in our experimental setup, or that the luxury nitrogen be allocated for chlorophyll production in new tissue (Azcón-Bieto & Talón 2008, Taiz *et al.* 2014). A decrease in the chlorophyll a/b ratio, such as what we observed for *Lepidium virginicum* and *Chloris gayana*, is an indication of an increased resource allocation to the light harvesting complex than to the photosystem reaction centers, leading to a reduced photosynthetic capacity, as it has been described for *Pseudoscleropodium purum* and *Rhytidiadelphus squarrosus* (Arróniz-Crespo *et al.* 2008, Lambers *et al.* 2008, Ochoa-Hueso *et al.* 2014).

The lack of response of F_v/F_m to nitrogen that was observed for most of the weeds suggests that its increased availability did not have a fertilization effect on photosynthesis, but, conversely, it did not impose stress either, including at the higher doses (Maxwell & Johnson 2000, DeEll & Toivonen 2011). The lack of response to high rates of N deposition can be associated with a more competitive behavior and greater tolerance of plants, as it has been found for invasive alien species (Lyons *et al.* 2013). In contrast, for the case of *C. pycnothrix*, the significant decrease of F_v/F_m for plants under 80 kg ha⁻¹ year⁻¹ was concurrent with the lowest carbon content for this species. This indicates a decreased use of light energy and photosynthesis under high N deposition rates, a stress that can lead to the decline of plant populations (Arróniz-Crespo *et al.* 2008).

Three conditions are required for developing an adequate biomonitor of environmental pollution: that the species has an ample distribution, that it is tolerant to an ample range of concentrations of the pollutant of interest, and that at least one physiological trait is sufficiently sensitive to respond in a “predictable” fashion to different levels of said pollutant (Markert *et al.* 2003). Indeed, the eleven ruderal weeds that were screened in the present study were selected based on their ample geographic distribution, their abundance in the region of interest, and

their apparent preference, or at least tolerance, for the elevated amounts of anthropogenic reactive nitrogen that are common in cities. In turn, the physiological parameters that were utilized have been shown to respond to nitrogen deposition both under experimental and field conditions. However, while most of the weeds displayed at least some biomonitoring potential, the mortality of four species was such that it precluded further physiological evaluation. This appeared to be more related to inherent characteristics of the plants, such as their ecological strategy of producing numerous seeds or low tolerance of the experimental handling, despite their successful proliferation in urban environments.

A field validation of the biomonitoring potential for *Bidens pilosa*, *Chloris gayana*, *C. virgata*, and *Pennisetum setaceum* is thus recommended, as 5-9 physiological traits of these species adequately responded to the experimental nitrogen deposition. All in all, the use of ruderal weed biomonitors, which are abundant in urban environments appears to be promising for characterizing nitrogenous pollution for consideration in integrative biomonitoring efforts.

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Supplementary material

Supplemental data for this article can be accessed here: <https://doi.org/10.17129/botsci.2789>.

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