

Ecological attributes promoting intra-continental range-expansion of a native annual forb triggered by intensified land-use

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Research Article

Keywords: alien, cyanogenic glycosides, distribution, invasive, neoflora, niche

Posted Date: June 1st, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-2999126/v1>

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Abstract

Intra-continental range-expansion of native species tracking human-induced environmental changes has become an important phenomenon, and recently has been suggested to be recognized as a distinct biogeographic category, namely neonative. However, it remains difficult to analyze how range-expansions are triggered by certain environmental changes and which biological and ecological species attributes are involved. We compared biological and ecological attributes and the performance in different test environments during the entire life cycle of a range-expanding neonative in Romania, *Xeranthemum cylindraceum*, with a congeneric resident native, *X. annuum*, through a series of controlled experiments. Both species proved to share several biological and ecological attributes, e.g. high potential for long-distance dispersal by multiple vectors, biochemical defence against herbivores by means of cyanogenic compounds, and surprisingly, they were found to have similar germination and performance niches throughout the range of climatic environments tested by us. Differences between the two species, a broader climatic and habitat niche, higher germination rate and viability of seeds can give explanation to the successful spread beyond the historical range of *X. cylindraceum*. However, based on shared biological attributes between the two species, which confer the potential to spread, and the considerably higher seed output found in the case of the resident native, *X. annuum* is equally prepared to expand its range. With changing climate and persisting human-induced disturbance, in this case intensive grazing by sheep, we may expect the spread of this species after passing the presumed migration lag phase.

Introduction

Human activities are causing an alarming biodiversity crisis, which is characterized by the decline of many native species (e.g. Rockström et al. 2009), and the accelerating spread of alien species around the globe (Seebens et al. 2017; Pyšek et al. 2020). Year by year, an increasing number of alien species establish themselves in the flora of Europe, with no signs of saturation (Seebens et al. 2017). Due to well-documented negative consequences of invasive alien species (inter-continental range expanding species) on biodiversity (Vilà et al. 2011), the intra-continental range-expansion of native species is also viewed as bearing potential risks. Thus, they raise concerns, especially among nature conservation authorities, scientists and even policymakers (e.g. Lesk et al. 2017; Adam et al. 2022).

Essl and coauthors (2019) posit that intra-continental range-expanding species tracking human-induced environmental change, the so-called 'neonative' species, should be treated as a separate case. They argue that there are substantial differences regarding the spread dynamics, biological and ecological features, and the impact caused on native biota between alien and neonative species. Alien species have been intentionally or unintentionally introduced by humans into new areas often far away from their native range, where they may naturalize and spread further. While neonative species expand their ranges through natural dispersal into new regions that are most often adjacent to their native range, tracking human-induced environmental change (Essl et al. 2019). There are only a few well documented case studies of neonative plants until the present, such as the northward range-expansion of *Lactuca serriola* in Central and Northern Europe (D'Andrea et al. 2009). Further, identifying specific biological and ecological attributes that favor successful range-expansion of certain species and the failure of others (e.g. resident natives), as well as the processes involved in the range-expansion is a key challenge for future research (Chen et al. 2011; Alexander et al. 2018; Essl et al. 2019).

Intra-continental range-expansion of native species has most often been linked to human-mediated climate warming. The range shift of plant and animal species along latitudinal and altitudinal gradients is widely accepted (e.g. Parmesan 2006; Gottfried et al. 2012; Brusca et al. 2013; Sillero et al. 2022). Under current global warming, climatic amelioration of formerly inappropriate regions may eliminate climatic (e.g. temperature) barriers of spread for thermophilous species. Thus, e.g. in Europe, plant species with a Mediterranean distribution are expected to expand northward. However, more complex patterns of range shifts, as well as drastic increases in abundance within species' ranges are also considered range-expansions (Burrows et al. 2011; Lenoir and Svenning 2015). Other human-induced disturbances, such as land-use change, over-harvesting and pollution, may also drive intra-continental range-expansion of species (Essl et al. 2019).

Contrary to the differences between alien and neonative species pointed out by Essl et al. (2019), drivers of spread, as well as traits and mechanisms explaining range-expansion success may, at least partly, overlap. Invoking invasion ecology theory is essential, since factors found to be important in affecting successful range-expansion of alien species might be helpful in identifying drivers of spread in the case of neonative species, especially in the establishment and landscape spread phase of this process (Theoharides and Dukes 2007; Wallingford et al. 2020). We argue that dispersal ability together with the availability of suitable habitats and dispersal vectors is essential for the landscape spread of range-expanding species in the new range irrespective of their alien or neonative status. A broader habitat niche (Hirzel and Le Lay 2008) may further increase the establishment and spreading success of species. In the same way, overcoming 'biotic resistance' of the recipient community is crucial for successful establishment, since pathogens, herbivores, or competition by other plants may all reduce spreading success (Richardson et al. 2000; Levine et al. 2004).

During the last decades, a striking range-expansion and increase in abundance of *Xeranthemum cylindraceum* Sm., an annual plant species of a Mediterranean original distribution, was observed in central Romania. This species colonizes disturbed xero- and meso-xeric grasslands, especially if they are heavily grazed by sheep. Like other Eastern European countries, Romania has undergone significant changes in land-use during the last decades (Feurdean et al. 2017). Agricultural policy promoted animal keeping, which caused increasing numbers of livestock, mainly sheep (Roman et al. 2019). Changes in the disturbance regime, namely the intensification of grazing, are presumably among the most important drivers of spread

in the case of *X. cylindraceum*. In the context of large-scale grazing disturbance, avoiding consumption by herbivores may increase competitiveness of the newcomers (e.g. Engelkes et al. 2008; Groscholz 2010). Cyanogenic glycosides, which are effective herbivore-deterrent secondary plant metabolites (Grime et al. 2007), have been identified in *X. cylindraceum* (Hübel et al. 1982; Schwind et al. 1990; Yulvianti and Zidom 2021). We hypothesize that successful defense against herbivores, including livestock, by means of cyanogenic glycosides may play a central role in the range-expansion of *X. cylindraceum*. This ability might provide a competitive advantage to this species in grazed plant communities. Further, high capacity for fast and massive germination as well as high performance across a wide range of environments may be key attributes linked to increasing range size, e.g. invasiveness of alien species (Richards et al. 2006; Ruprecht et al. 2014).

Comparisons between neontic and native species might elucidate which traits are likely responsible for the successful range-expansion of species belonging to the first group. Using congeneric pairs of species with similar life form and original distribution may further increase the reliability of comparative studies of this type.

In this study, we compare biological and ecological attributes and performance during the entire life cycle and along environmental gradients of the range-expanding *X. cylindraceum* to its resident native congener, *Xeranthemum annuum* L. We seek to unravel causal factors underlying the potential to expand of *X. cylindraceum* compared to its close relative. We hypothesize that (1) high dispersal ability, (2) broad climatic niche and habitat range, (3) fast and massive germination, (4) consistently high performance throughout a wide temperature range make *X. cylindraceum* more suited for expansion into new areas compared to its congener. In addition, (5) effective herbivore defense helps this species cope with the main disturbance type of the new range, in this case high grazing pressure.

Material and methods

Study species

Both studied *Xeranthemum* species, *X. cylindraceum* Sm. and *X. annuum* L. (Asteraceae), are winter annuals, i.e. seeds germinate in autumn, the seedlings overwinter, and the plants start to flower in June-August (depending on climatic conditions). Fruits are dispersed during August-October. The fruits (achenes) have 10–15 (*X. cylindraceum*) or 5 (*X. annuum*) pappus-scales, and are relatively big (seed mass *X. cylindraceum* = 0.59 mg, *X. annuum* = 0.11 mg; data on seed mass are the results of our own measurements of 3 × 100 seeds per species, averaged, and expressed as the mass of a single seed). Cyanogenic glycosides have been identified in fruits of *X. cylindraceum* and roots of *X. annuum* (Schwind et al. 1990; Orhan et al. 2016). Both species occur in grasslands, shrublands and forest openings in Romania, with *X. annuum* being present in ruderal habitats as well (Sârbu et al. 2013).

Xeranthemum cylindraceum has a Mediterranean, while *X. annuum* has a Pontic-Mediterranean distribution (The Euro + Med PlantBase 2022). They are considered native species in Romania, with occurrences mostly in the southern and south-eastern parts of the country (Grecescu 1898; Săvulescu and Nyárády 1964; Sârbu et al. 2013). In the first half of the 19th century, *X. cylindraceum* was present only in the warmest, south-western part of the Transylvanian Basin (Simonkai 1886), while by the end of the century it has also been recorded in the northern-central part of this region as well (herbarium data). In the 20th century, it was considered a rare species in Transylvania (Nyárády 1943; Soó 1944), while more and more occurrences of this species appeared in literature sources (floristic surveys and vegetation plots) and herbarium data from the northern and central part of this region until the end of the century (see Online Resource 1, Fig. 1). Thus, *X. cylindraceum* is considered a range-expanding species in Transylvania with significant range expansion to the north during the last 150 years, and range-filling during the last 60 years (see Online Resource 1, Fig. 1). Contrary to this, *X. annuum* is constantly restricted to the south-western part of the Transylvanian Basin, and it has a stable range in there from the 19th century until the present (see Online Resource 1, Fig. 1).

Seed dispersal experiments

We compared the potential for animal and wind dispersal of *X. cylindraceum* and *X. annuum* seeds with that of 88 species listed as alien in Romania, of which seeds could be collected by our research team (see Online Resource 2). Alien species were classified into casual or naturalized alien species which are not invasive (n = 50), and invasive alien species (n = 38) based on the Romanian national checklist of alien plant species (Anastasiu and Negrean 2009). Seeds of the alien species have been collected in 2021 from Romanian populations, while seeds of the two *Xeranthemum* species originated from our transplant experiment (see below).

Epizoochory

Animal dispersal (epizoochory) by sheep was tested as the ability of propagules to attach to sheep fur. A wooden table 40 × 50 cm was covered with the fur and pressed to a sheet of paper with propagules spread over it. The number of attached propagules was counted after three circular movements of the frame pressed to the paper (Moravková et al. 2010). 100 propagules were randomly selected and used to make four replicates of 25 one-seeded fruits. The potential for epizoochorous propagule dispersal by sheep was expressed in percentages.

Wind dispersal

To assess the wind-dispersal ability (anemochory) of the above-mentioned seeds, we measured terminal velocity, i.e. the maximum rate of fall, calculated by dividing the height of the fall by the duration of seed descent (Andersen 1992). We used a 20 cm diameter tube to exclude the air

movement of the room, which was applied on an opened box to achieve the visibility of seeds at the end of their fall. We placed a Petri-dish with a hole above the tube, in which we stored the seeds (10 seeds per each species) before dropping them in the tube.

Using a Canon 60D camera (NTSC, 60 frame/sec resolution), we recorded the releasing moment of the seed, and also the sound when it comes to rest (we placed an aluminium foil under the tube to amplify the sound of the falling seeds). With a video editor program, we noted the exact frame when the seed fell through the hole of the Petri-dish, and we analyzed the sonogram to find the moment (i.e. the frame) when the seed finished falling. We transformed the number of frames recorded during the fall to secundum, and we divided the total length of the fall (1.47 m) with the duration of fall.

Climatic and habitat niche breadth

To estimate the climatic niche breadth and niche overlap of the two *Xeranthemum* species, we used dynamic range boxes, a robust nonparametric approach to quantify size and overlap of n-dimensional hypervolumes, where each niche component represents one dimension, while no assumptions about the underlying distributions are required (Junker et al. 2016). Distribution (occurrence) data of the two species was extracted from Global Biodiversity Information Facility (GBIF) database, using the *gbif* function of *dismo* package in R (<https://www.gbif.org/>; Hijmans et al. 2021). To exclude occurrence duplicates and avoid oversampling of particular locations, we calculated mean coordinates of occurrences within the cells of a 30 × 30 seconds (~ 1 km²) grid for each species separately, permitting the occurrence of two species within one grid cell while eliminating more than one occurrence of the same species within a cell. After this procedure, we obtained a total of 2819 observations, 1578 for *X. cylindraceum* and 1241 for *X. annuum*. Climatic data was extracted for each occurrence point from the 19 Bioclimatic variable rasters from the WorldClim database (Fick et al. 2017; <https://www.worldclim.org/data/worldclim21.html>). Because highly correlated variables might cause inaccurate estimation of hypervolumes (Junker et al. 2016), we excluded one of two variables where absolute pairwise correlation was high ($r > 0.75$), and limited our selection to 11 climatic variables for niche size and overlap estimation (Cai et al. 2021): annual mean temperature (Bio1); mean diurnal range (mean of monthly (max temp - min temp); Bio2); isothermality (BIO2/BIO7×100; Bio3); temperature annual range (BIO5-BIO6; Bio7); mean temperature of wettest quarter (Bio8); mean temperature of driest quarter (Bio9); precipitation of driest month (Bio14); precipitation seasonality (coefficient of variation) (Bio15); precipitation of wettest quarter (Bio16); precipitation of warmest quarter (Bio18); precipitation of coldest quarter (Bio19). We estimated niche breadth (expressed as hypervolume size) and overlap (expressed as hypervolume overlap) with the *dynRB_VPa* function from the R package *dynRB* using the “gmean” aggregation function, which permits comparison of hypervolumes between different variable sets (Junker et al. 2016). Hypervolume size and overlap ranges from 0 to 1, values closer to 0 indicate a narrow niche and little overlap in niche space, while values closer to 1 indicate a wide niche and high overlap in niche space between two species (Junker et al. 2016). Furthermore, the original dimensions of the selected 11 climatic variables were replaced with principal components (Junker et al. 2016; Cai et al. 2021).

In order to determine the habitat range of the two species, we extracted vegetation data (phytosociological relevés) with the presence of *X. cylindraceum* (173 relevés) or *X. annuum* (861 relevés) from the Romanian Grassland Database, containing 43.249 vegetation plots from open habitats of Romania (<https://edgg.org/index.php/databases/Regional-databases>). Due to spatially non-homogeneous distribution and unequal numbers of relevés per species, after a random resampling performed in JUICE 7.1 (Tichý 2002), we analyzed 93 relevés of each species to have an equal number of observations. After that, we classified vegetation plots in EUNIS habitat categories using the expert system EUNIS-ESy (Chytrý et al. 2020).

Germination trial

Seeds of the two *Xeranthemum* species were collected in bulk from one population each in September 2019 to use them in a germination and a transplant experiment. Seeds of *X. cylindraceum* were collected at Inucu, Romania (coordinates: N46.843641, E23.252089, 570 m elevation), while seeds of *X. annuum* were collected at Lancrăm, Romania (coordinates: N46.013341, E23.556629, 226 m elevation).

In February 2020, the seeds of both study species were spread on a double layer of filter paper in Petri dishes. We used 25 seeds per Petri dish. Seeds were incubated at four constant temperatures of 5, 10, 15, and 20°C and one diurnally fluctuating temperature (25/15°C) with a 12 hr light and 12 hr dark photo-cycle in climate chambers (SanyoMLR-352; Sanyo/Panasonic Healthcare Co., Ltd, Japan.) in the Laboratory of the University Botanical Garden of Cluj-Napoca. We had five replicates per each treatment and species combination. The Petri dishes were placed in transparent plastic bags sealed with Velcro to minimise water loss from evaporation. The dishes were regularly watered with distilled water to optimise moisture conditions. The location of the dishes in the chamber was randomised at the beginning of the experiment and at every census of the germinated seeds. Germinated seeds, i.e. those seeds showing radicle protrusion, were counted and removed every second or third day. The experiment lasted for three weeks. At the end of the experiment, we calculated the proportion of seeds germinated. Viability of non-germinated seeds was tested with a 1% tetrazolium chloride (2,3,5-triphenyltetrazolium chloride) solution, thus we could express the average proportion of germinated, dormant and dead seeds through all the temperature conditions for both species.

To describe germination characteristics, the following indexes were calculated:

TOPT, the optimal temperature for germination, was calculated as weighted average of germination rates over all constant temperatures, according to Olff et al. (1994) and Hölzel and Otte (2004): $[(5 P_5 + 10 P_{10} + 15 P_{15} + 20 P_{20}) / (P_5 + P_{10} + P_{15} + P_{20})]$ in which P_5 was percentage germination at 5°C, P_{10} percentage germination at 10°C, and so on.

*L*MAX, the maximum germination rate, was calculated as the highest germination rate under one of the temperature treatments by the end of the trial; *T*MAX, the temperature at which the highest germination rate was attained by the end of the trial; *LOW* was the lowest constant temperature with at least 5% germination; *HIGH* was the highest constant temperature with at least 5% germination (Hölzel and Otte 2004). We also calculated time to germination in days for each sample following Tielbörger and Prasse (2009): $\Sigma(i \times n_i) / \Sigma n_i$, with *i* being the number of days since the beginning of the experiment and *n_i* being the number of germinating seeds (radicle emergence) on day *i*, and compared germination speed between the two species.

Transplant experiment

In September 2019, we sowed 25 seeds of both species individually in 1-L pots (c. 10 x 10 x 10 cm) on the top of the soil surface. The pots were filled with a 2:1 mixture by weight of commercial potting soil and sand. To each pot 0.05 g of ammonium-nitrate and 0.025 g of nutrient mixture (20% P2O5, 44% K2O, 0.05% Fe, 0.025% Mn, 0.008% Zn, 0.006% Cu, 0.02% B, 0.0035% Mo) was added before the beginning of the experiment in order to ensure that there was no shortage in any of the necessary nutrients. 48 pots (24 per species), protected by wooden frames covered by a polyethylene sheet, were placed in the University Botanical Garden at Cluj-Napoca, Romania. After the seeds germinated, we counted the seedlings and prepared four sets of six pots per species to be transferred to four experimental sites. There was no difference in seedling numbers allocated to the experimental sites in any of the species (results of one-way ANOVA with site as explanatory variable: *X. cylindraceum*, MS = 3.83, df = 3, F = 1.45, p = 0.259, *X. annuum*, MS = 2.04, df = 3, F = 0.14, p = 0.932).

We selected four experimental sites (common gardens) in Romania based on elevation, in order to represent a climatic gradient (see Online Resource 1, Table 1). The climatic gradient covered mean annual temperatures between 5.1 and 10.2°C, and mean annual precipitation between 600 and 833 mm (for the period 1970–2000; WorldClim database, Fick and Hijmans 2017). *X. cylindraceum* spontaneously occurs in the surroundings of two of the experimental sites: Cojocna and Inucu, while the transplant sites are outside of the actual range of *X. annuum*. Sites were equipped with temperature data loggers (Hobo Pendant UA-001-64, Onset Computer Corporation, Bourne, MA). Loggers were placed on the soil surface and covered by a clay pot to prevent loggers from direct solar radiation. We monitored soil surface temperature on a bihourly basis.

Seedlings were transferred to the experimental sites between 9 and 16 October 2019. Pots were buried in the soil in a random arrangement. Seedlings overwintered and adult plants were grown in these buried pots in the experimental sites, allowing them to experience natural changes in seasonal temperature and precipitation. Since neither seedlings nor adult plants were protected against insects and snails or slugs, their survival and growth might have been influenced by herbivore activity as well. Seedling survival was checked on 10 March 2020 by counting all the living seedlings in the pots. On 10 May 2020, we pulled out all the seedlings but one per pot. The roots of the seedlings were washed free of soil, their biomass was dried at 60°C for 48 h, and above- and below-ground biomass of 10 randomly selected seedlings was weighed. One seedling per each pot was let to grow and reach the adult stage.

Between 18 July and 19 August 2020, we harvested all adult plants, counted the number of capitula produced by each plant (three stages were noted separately: bud, flowering, and fruiting stage), cut the biomass above-ground, and washed the roots free of soil. Above- and below-ground biomass was dried for 48 h at 60°C and weighed. Time of harvesting was adjusted so as the plants to reach the same phenological stage at the four experimental sites. Since we wanted to reduce the risk of spreading the introduced species seeds at and around the experimental sites, we took care of adult specimens not to disperse their fruits before the harvesting. Plants were thus harvested at peak flowering, having only a few fruiting capitula. The sequence of harvesting was: 18 July at Satu-Mare, 9 August at Cojocna, 12 August at Inucu, and 19 August at Răchițele.

We calculated several climatic variables based on the bihourly soil surface temperature data registered by temperature loggers. Mean soil surface temperature during the day (°C) and mean soil surface temperature during the night (°C) were calculated separately for each life stage of grown plants in the four transplant sites. In addition, we quantified the overall thermal stress during the period of early seedling stage expressed by freezing period length (FPL, the number of days with daily soil surface temperatures < 0°C) and freezing degree days (FDD, the sum of daily soil surface temperatures < 0°C), which summarize the harshness of thermal conditions posed to seedlings. In the same way, we quantified the overall favorable thermal conditions during the period of early and late seedling stages expressed by growing period length (GPL, the number of days with daily soil surface temperatures > 5°C) and growing degree days (GDD, the sum of daily soil surface temperatures > 5°C), which indicate the length of the growing period and the amount of energy available for plants. These four indices summarize soil thermal conditions experienced during the unfavorable and the favorable period for seedling growth.

In the case of early seedling stage, we analysed seedling survival during the winter, using the number of dead seedlings and the total number of seedlings at the start of the experiment as response variables against the interaction of species and locality, with binomial link function (logistic regression). While in the case of late seedling stage, total biomass of 10 randomly selected seedlings was compared among the transplant sites and the two species by applying a linear model. In one of the sites, Satu-Mare, there were only three seedlings left by slugs for biomass measurements.

In the case of adult stage plants, we analysed four response variables: total biomass, root-shoot ratio, the number of capitula, and the number of seeds per fruiting capitula. Since we worked with annual plant species in this experiment, biomass, the number of capitula, and the number of seeds per fruiting capitula can be considered as measures of overall plant performance and fitness. We used linear models with species, site and their

interaction term as explanatory variables. The first three variables were log transformed prior to the analyses, while in the case of seeds per fruiting capitula, we examined two separate linear models for each species, because of the bimodal character of the variable. For post-hoc comparisons among sites and species, we used the *emmeans* package (Lenth 2021).

Determination of total quantity of cyanogenic glycosides in fruits, seedlings and shoots

Tissue content in cyanogenic glycosides was evaluated by determination of enzymatic hydrolysis of the glycosides and subsequent determination of the released hydrogen cyanide using barbituric acid-pyridine reaction and fluorescence detection. For this purpose, 50 fruits (achenes), 30 late stage seedlings (7-month-old) and six adult plant individuals of *X. cylindraceum* and *X. annuum* were used. Aboveground plant parts were dried for 48 h at 60°C, and then stored at room temperature for several months prior to the chemical analyses. In addition to these, freshly collected seedlings of *X. cylindraceum* were also used for the chemical determination. The fresh plant material (seedlings) was lyophilized and stored in the refrigerator until used. A randomly selected, representative part of the plant material – separately for fruits, seedlings, leaves and stems – was milled to fine particles using 5 mm metallic balls and a Retsch Mill MM 400 tissue lyser. An exact mass of this fine powder was weighted using an analytical balance – in the range between 20 and 100 mg – and was transferred to a 20 mL screw neck glass vial alongside a 5 mm stirrer bar and a 0.5 mL capless tube containing 100 µL 1 M sodium hydroxide. A volume of 500 µL solution of 50 mM sodium citrate buffer pH 5.90 containing β-glucuronidase from *Helix pomatia* (12 U mL⁻¹) was added to the plant powder. After the addition of the buffer solution, the vials were immediately tightly capped and sealed with parafilm and magnetically stirred for homogenization for half an hour, then kept steady at room temperature for 24 hours.

Similar approach was conducted on a series of standard solutions of pure amygdalin. The enzymatically released hydrogen cyanide was trapped in the sodium hydroxide solution following its determination using barbituric acid-pyridine analytical procedure, as previously described by Gleadow and Woodrow (2002) and references therein. Briefly stated, an exactly known volume of the sodium hydroxide solution containing trapped hydrogen cyanide was transferred in a 1 mL tube alongside 330 µL water, neutralized with a double volume of 0.5 M acetic acid followed by the addition of 100 µL chloramine T 1% solution and 300 µL solution containing barbituric acid (30 mg mL⁻¹) and pyridine (20% v v⁻¹). The sample mixture from the plant samples or analytical standards was immediately vortexed and incubated 15 minutes at room temperature, then their fluorescence signal was read at 610 nm using a SilverNova-CuvF-SL1LED fluorescence system while exciting with a LED at 502 nm. By the aid of a calibration curve constructed using amygdalin as standard, the analytical signal was converted to equivalent cyanide content and the values were expressed as µg g⁻¹ dried material.

Total cyanogenic glycosides content of *X. cylindraceum* and *X. annuum* leaves and stems was compared using a linear model with species, plant part and their interaction term as explanatory variables. Variables were log transformed prior to the analyses. For post-hoc comparisons among species and plant parts, we used the *emmeans* package (Lenth 2021). All analyses were performed in R 4.1.1. (R Core Team 2021).

Results

Dispersal ability

Based on our seed dispersal experiments, the seeds of *X. cylindraceum* had a higher probability for epizoochorous dispersal by sheep (54.5%) than those of *X. annuum* (39.5%). As compared to the two groups of alien species, which can be found in Romania, i.e. casual or naturalized alien species which are not invasive, and invasive alien species, both *Xeranthemum* species may be classified as very successfully dispersed by means of epizoochory (Fig. 1A). In respect of wind dispersal, in comparison to the two groups of alien species, both *Xeranthemum* species were proved to have a medium potential for anemochorous seed dispersal, with *X. annuum* seeds having lower velocity thus a higher probability for wind dispersal than *X. cylindraceum* (Fig. 1B).

Climatic niche breadth and habitat range

The climatic niche of *X. cylindraceum* was found to be broader (hypervolume size estimate = 0.60) than that of *X. annuum* (0.47). The niche overlap between the two species was moderate: estimate for the overlap between *X. cylindraceum* and *X. annuum* was 0.40, while between *X. annuum* and *X. cylindraceum* was 0.30. The asymmetry in overlap was the result of the different hypervolume sizes for the two species.

Based on an equal number of observations, *X. cylindraceum* occurred in a wider range of habitat types in Romania, since it has been found in 14, while *X. annuum* in 10 EUNIS habitat types. Continental dry grassland (true steppe) was the most preferred habitat type in the case of both species (see Online Resource 1, Table 2). Regarding differences between the habitat suitability of the two species, *X. cylindraceum* occurred more often in semi-dry or mesic habitats, while *X. annuum* in dry and man-made habitats (see Online Resource 1, Table 2).

Seed germination

Seeds of both species germinated without any pretreatment, e.g. cold-wet stratification or scarification, and rapidly, i.e. after 3.4 ± 1.6 (*X. cylindraceum*) and 3.8 ± 1.9 (*X. annuum*) days of incubation on average, in climate chambers. Concerning the breadth of the germination niche, seeds of both species germinated well (above 50% germination rate) under a wide range of temperatures. Temperature requirements for germination were similar or identical between the two species (*TOPT*: *X. cylindraceum* = 12.47°C, *X. annuum* = 12°C; *TMAX*: *X. cylindraceum* = 10.15°C, *X. annuum* = 10°C; *LOW*: *X. cylindraceum* = 5°C, *X. annuum* = 5°C; *HIGH*: *X. cylindraceum* = 20°C, *X. annuum* = 20°C). The maximum germination rate, however, was considerably higher in *X. cylindraceum* (*LMAX*: *X. cylindraceum* = 99.2%, *X. annuum* = 76%). The lower germination success observed in the case of *X. annuum* can be attributed to the substantially lower seed viability of this species compared to *X. cylindraceum* (Fig. 2). The proportion of dormant, non-germinating seeds was found to be very small by the end of the germination experiment in the case of both species (*X. cylindraceum* 0.8–1.6%, *X. annuum* 1.6–8.8%) and through all the temperature conditions (Fig. 2).

Performance during the life cycle in test environments

Climatic conditions in test environments

There were marked differences in mean soil surface temperatures during the experimental period between the transplant sites (between 6.8 and 9.4°C). The Inucu site was the coldest among all the transplant sites during the winter period (early seedling stage) with 3.6°C mean soil surface temperature during the day, 1.1°C during the night and 60 days of freezing temperatures (see Online Resource 1, Table 1). During the late seedling stage, the Răchițele site was the coldest, with 9.8°C mean soil surface temperature during the day and 2.7°C during the night, and Cojocna the warmest, with 11.7°C mean soil surface temperature during the day and 6.1°C during the night (see Online Resource 1, Table 1). During adult plant development, the Răchițele site was the coldest, with 14.8°C mean soil surface temperature during the day and 11.8°C during the night, and Satu Mare the warmest, with 18.9°C mean soil surface temperature during the day and 17.9°C during the night (see Online Resource 1, Table 1).

Seedling survival and development

Overall, the rate of seedling survival was lower in *X. annuum*. Seedlings of *X. annuum* survived the winter at a smaller rate at two sites, Satu Mare and Cojocna, while there was a constantly high seedling survival in the case of *X. cylindraceum* at all the four transplant sites, with a slightly lower seedling survival at Răchițele compared to Inucu (Table 1, Fig. 3A). The owners of the gardens at Satu Mare and Cojocna observed the presence of slugs during early spring, while no slug presence was observed in the other two transplant sites.

There was no difference in the biomass of 7-month-old seedlings either between the four transplant sites or between the two *Xeranthemum* species (Table 1). Seedlings of the two species developed equally well during early spring in each of the four transplant sites.

Adult development and plant fitness

We found a striking difference in the total biomass of adult specimens at harvest between the four transplant sites (Table 1, Fig. 3B), with plants of both *Xeranthemum* species grown at the Răchițele site developing much bigger (*X. cylindraceum* by 88.2%, *X. annuum* by 80.7%) than in the other three sites on average (Fig. 3B). Both *Xeranthemum* species had the lowest root-shoot ratio at the Răchițele site (Fig. 3C). In addition, *X. cylindraceum* had a higher root-shoot ratio than *X. annuum* through all the transplant sites (Table 1, Fig. 3C).

All specimens of the two study species flowered during the experimental period at each transplant site, although there was a huge variance in the number of capitula between specimens (*X. cylindraceum* between 2 and 291, *X. annuum* between 4 and 166). The number of capitula did not differ between the two *Xeranthemum* species, but it differed between the transplant sites, being the highest at the Răchițele site in both species (by 83% higher than in the other three sites on average; Table 1, Fig. 3D).

Seed number per fruiting capitula was found to be much higher in *X. annuum* (82.3 ± 30.0) than *X. cylindraceum* (8.9 ± 2.8). Site influenced the number of seeds per capitulum in both species, with *X. cylindraceum* having a larger number of seeds per capitulum at Răchițele than at Cojocna, with Satu-Mare and Inucu in between ($F = 6.51$, $p = 0.010$). *X. annuum* also had a larger seed number of seeds per capitulum at Răchițele than at Cojocna and Satu-Mare, with Inucu in between ($F = 9.11$, $p = 0.001$).

Table 1

Effect of site (four transplant sites, for details see see Online Resource 1, Table 1) on the survival rate between 16 October 2019 and 10 March 2020 of early stage seedlings, on the biomass of late stage seedlings (on 10 May), on the final biomass, root-shoot ratio and number of capitula of adult specimens at harvest (between 18 July and 19 August 2020) of *X. cylindraceum* and *X. annuum*.

Source of variation	Survival of seedlings			Biomass of seedlings			Biomass of adults			Root-shoot ratio			Number of capitula		
	df	chisq	p	SS	F	p	SS	F	p	SS	F	p	SS	F	p
Site	3	110.7	< 0.0001	1.13	1.76	0.1645	46.14	29.83	< 0.0001	6.69	8.83	< 0.001	30.68	14.82	< 0.0001
Species	1	128.6	< 0.0001	0.26	1.2	0.2776	1.51	2.93	0.096	1.74	6.88	0.0127	0.44	0.63	0.431
Site x Species	3	63.68	< 0.0001	0.77	1.2	0.3186	1.12	0.72	0.546	0.15	0.19	0.899	1.34	0.65	0.589
Residuals	36			13.9			18.05			9.09			24.83		

Biochemical defense against herbivores

All analysed plant parts or developmental stages of both study species contained cyanogenic glycosides. A very high total quantity of cyanogenic glycosides was found in the fruits of *X. cylindraceum* ($507.20 \mu\text{g g}^{-1}$), while the total quantity of these compounds was low in fruits of *X. annuum* ($0.75 \mu\text{g g}^{-1}$). The total quantity of cyanogenic glycosides in dried, late-stage seedlings of *X. annuum* ($0.24 \mu\text{g g}^{-1}$) was higher than in seedlings of *X. cylindraceum* ($0.12 \mu\text{g g}^{-1}$; in fresh, lyophilized seedlings, this value was approximately three times higher). In adult plants, the total quantity of these compounds was higher in leaves than in stems in both species, and in leaves of *X. annuum* the total quantity of these compound was found to be higher than in leaves of *X. cylindraceum* (Fig. 4).

Discussion

Dispersal ability of propagules and dispersal vectors

Dispersal potential and the presence of dispersal vectors are very important prerequisites for successful range-expansion in plants (Richardson et al. 2000; Theoharides and Dukes 2007). Our results are the first to quantify epizoochorous and anemochorous seed dispersal in *X. annuum* and *X. cylindraceum*. Based on the protocol of Moravková et al. (2010) followed by us, seeds (achenes) of both species proved to have an outstanding dispersal ability in animal fur even in comparison to a large set of alien invasive species. Thereby, the propagules of both species are well-adapted to be dispersed over long distances by native mammals, and equally well by shepherded livestock, e.g. sheep. Furthermore, seeds of both species may also be dispersed by wind, which means that they have multiple dispersal vectors and, thus, a high potential for spread.

Climatic constraints on plant survival and reproduction

We tested the performance through the entire life cycle and over a wide range of temperature conditions of the currently expanding *X. cylindraceum* and the congeneric resident native *X. annuum* by a series of experiments. Since both species are short-lived, seed germination, seedling survivorship and development are crucial for successful reproduction.

The germination trial revealed that the seeds of both *Xeranthemum* species germinate rapidly and without any pretreatment. Fast germination without any special requirements for germination has been mentioned as important determinant of invasiveness (Goodwin et al. 1999; Sakai et al. 2001). The breadth of the germination niche ('regeneration niche' sensu Grubb 1977) may influence the breadth of the ecological niche and by this means the geographical range of a species (Donohue et al. 2010). Further, germination requirements are considered among key factors explaining the decline, persistence or spread of plant species under changing disturbance regimes (Schütz 2000). Based on our results, both species have a broad germination niche and the potential to successfully establish next generation specimens in a variety of regions with average autumn soil surface temperatures between 5 and 20°C. In respect of between species differences, *X. cylindraceum* proved to have a higher germination rate (*LMAX*). This, together with a wide germination niche may increase the potential to establish new populations outside the actual range.

Our field transplant experiment was designed to represent a climatic gradient based on the current and beyond the current Transylvanian distribution of the two study species. From the four transplant sites, the Inucu site posed the harshest conditions for seedling survival during the winter, since mean soil surface temperatures during the night and the day were the lowest as well as FPL (freezing period length) was the longest and FDD (freezing degree days) was the highest from all the transplant sites. Seedlings of both species were found to be adapted for cold tolerance, since they survived at a high rate under the entire range of winter temperatures tested by the transplant experiment (mean soil surface temperature of coldest quarter between -0.7 and 1.8°C). We note, however, that winter temperatures in the year of our experiment have not been among the harshest (multiannual mean temperature of coldest quarter ranges between -3.7 and -0.3°C ; see Online Resource 1, Table 1).

However, seedlings of *X. annuum* showed up to be more vulnerable in front of herbivore attacks, since we found a considerable loss among early-stage seedlings at the two warmest transplant sites caused by slugs. In a review by Hanley (1998), molluscs have been argued to be the most important selective invertebrate herbivores in temperate grasslands. Molluscs prefer to consume herbaceous plants during their early ontogenetic stages (Barton and Koricheva 2010), and a herbivore attack in the seedling stage has fatal consequences on plant fitness and survival (e.g. Hanley 2012; Barton 2013). *Xeranthemum annuum* proved to be less defended against herbivores in the establishment phase, which might restrict its colonisation into new sites with a winter- or early spring abundance of seedling-consumers. In other words, unlike the range-expanding *X. cylindraceum*, *X. annuum* seems to face a bottleneck mediated by seedling-consumers in the establishment phase, which might have major consequences on the population persistence and spread of this species. Except for the effect of slugs on *X. annuum* seedlings during the early spring period, no other herbivore attack was observed on seedlings or adult plants of the two *Xeranthemum* species during the whole experimental period.

Intriguingly, the large climatic differences between the transplant sites during the late seedling stage had no influence on seedling development, while climatic conditions in summer affected adult development. The two *Xeranthemum* species did not exhibit superior performance under temperature regimes corresponding to their current range in Transylvania, but surprisingly, under the harsher temperature conditions prevailing outside of their actual range in this region (Răchițele site). Mean annual precipitation was the highest, in addition, the summer period was the coolest from all the four transplant sites at Răchițele, resulting in lower evaporation rate thus an elevated water availability to plants, and ultimately in higher biomass of specimens grown at this site. Patterns in the number of capitula as well as in seed number per fruiting capitulum between the sites strengthen our results found for total biomass. The limiting role of water availability rather than higher summer temperatures in developing larger biomass, more inflorescences and ultimately reaching higher fitness in both *Xeranthemum* species is supported by the lowest root-shoot ratio of specimens of both species grown at Răchițele, compared to the other transplant sites. A lower root-shoot ratio indicates that water or soil nutrients are less limiting to plant growth, thus specimens can invest more resources into their above-ground parts at the expense of their roots. The higher root-shoot ratio of *X. cylindraceum* compared to *X. annuum* may denote that it is more limited by water availability. This is in accordance with the higher frequency of *X. cylindraceum* in mesic habitats in Romania (see our results above). Our experimental results in test environments of reduced competition with other plant species (common gardens) point out that autecological preferences of the study species do not fully overlap with their natural distribution and the range of habitats in which they occur (realized range; see also Booth 2017). Higher competitive pressure in colder and moister thus more productive habitats may hamper the establishment and successful reproduction of these two winter annual species.

Contrary to the larger climatic niche of the range-expanding *X. cylindraceum*, which we found, the two congeners had a very similar performance through the different climatic conditions of the transplant sites. However, our experimental results are based on only one population per species, thus results should be interpreted with caution.

Biochemical defense against herbivores

Tissue content in cyanogenic glycosides in shoots of both *Xeranthemum* species may ensure a protection against herbivores and pathogens in their adult stage. Based on our results referring to seedling content in these compounds, the synthesis of cyanogenic compounds is already turned-on in both species at the late seedling stage. However, it may be absent in the very early seedling stage of the resident native, which caused losses by slug herbivory in two of our transplant sites among young seedlings of *X. annuum*. In addition, we detected a huge quantity of cyanogenic compounds in seeds of *X. cylindraceum*. Since large seeds are highly susceptible to seed predation (Reader 1993; Maron et al. 2012), successful protection of seeds by means of chemical compounds may give an advantage to this annual species in recruiting into plant communities in a new range. Based on all these, *X. cylindraceum* can resist the pressure by consumers and pathogens during the entire life cycle, and this attribute may have an important contribution to the success of this species, especially in habitats under high herbivore pressure, including grazing lands. Like invasive species, successfully or rapidly expanding neontic species can possess characteristics that are ecologically novel in their new range (Saul and Jeschke 2015). The number of vascular plant species (between 2500–3000; Hegnauer 1977; Zagobelny et al. 2008), which are known to contain cyanogenic compounds, and the ability to liberate toxic volatile HCN to defend themselves against herbivore or pathogen attacks (Conn 1980), is relatively low, thus this type of chemical defense may represent a biochemical “novel weapon” (Callaway and Ridenour 2004; Schaffner et al. 2011).

Conclusions

The two annual congeners studied by us, *X. cylindraceum* and *X. annuum*, proved to share essential biological attributes relevant for range-expansion under environmental change, such as propensity of long-term dispersal by multiple vectors, an outstanding ability for epizoochorous seed dispersal and avoiding consumption by herbivores by means of biochemical defense, which confer the potential for range-expansion to both species. The currently expanding species (*X. cylindraceum*) has a broader climatic and habitat niche, higher germination rate and higher seed viability compared to its resident native congener. However, *X. annuum* has a considerably higher seed output, which may counterbalance the pre- and post-dispersal seed losses caused by predation.

Based on our experimental investigations on single populations of the two *Xeranthemum* species, both were found to tolerate a wide temperature range, and to be pre-adapted to harsher climatic conditions occurring in areas beyond the limits of their current range. Climate effect on plant

development was indirect through the early spring activity of herbivores during the early seedling stage, it had no influence during the late seedling stage, but it became decisive in influencing fitness of adult plants through water availability.

In this sense, we consider that both *Xeranthemum* species are well prepared to spread in new areas with a changing climate, where human-induced environmental perturbations, e.g. large-scale intensive grazing by sheep, prevail. We cannot exclude the possibility of *X. annuum* currently being in a migration lag, thus showing itself as a range-expanding species in the future (Corlett and Westcott 2013).

Declarations

Acknowledgements

We are very grateful to Ildikó Jankó-Szép, Kinga Csoltkó, Zsuzsi Máthé and Péter L. Pap for their help during the transplant experiment, to Norbert Imreh for assistance during the germination trial, to Dorottya Sándor for assistance during plant measurements, to Rita Tímár and Mátyás Sztojka for their valuable suggestions and contribution during the whole project. This study was funded by the Romanian Ministry of Research, Innovation and Digitization [CNCS/CCCDI – UEFISCDI, project PN-III-P1-1.1-TE-2019-0414]. Franz Essl was supported by the AgriWeedClim project by the Austrian Climate Research Program [FA772033 "AgriWeedClim"].

Funding

This study was funded by the Romanian Ministry of Research, Innovation and Digitization [CNCS/CCCDI – UEFISCDI, project PN-III-P1-1.1-TE-2019-0414]. Franz Essl has received research support from the AgriWeedClim project by the Austrian Climate Research Program [FA772033 "AgriWeedClim"].

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Eszter Ruprecht and Zsombor Miholcsa conceived of the ideas and design of the study. *Data collection and analysis were performed by Eszter Ruprecht, Zsombor Miholcsa, Augustin C. Moț, Beatrix Balaji and Constantin Mardari. The first draft of the manuscript was written by Eszter Ruprecht, Franz Essl and Zsombor Miholcsa and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.*

Data Availability

Data about the distribution and habitat preference of *Xeranthemum cylindraceum* and *Xeranthemum annuum* are available from the Romanian Grassland Database. Data may be accessed upon request from the Custodian of the database (<https://edgg.org/index.php/databases/Regional-databases>). Data about dispersal traits are presented as Online Resource 2 (ESM_2.xlsx). Other *datasets generated and analysed during the current study are available from the corresponding author on reasonable request.*

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Figures

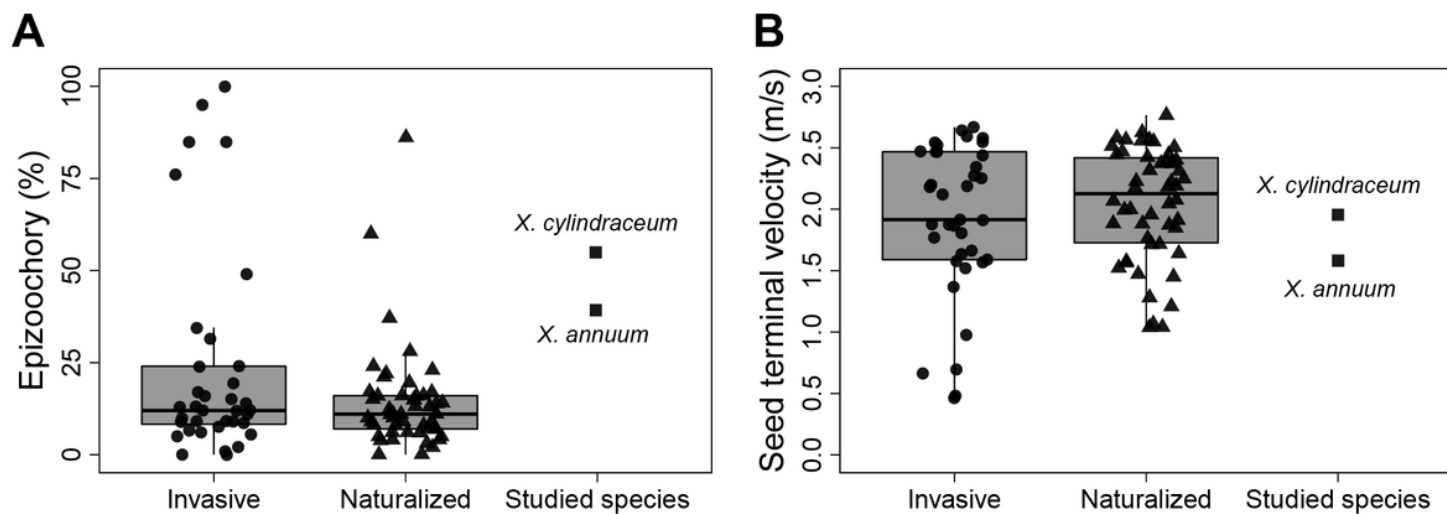


Figure 1

The potential for epizoochorous propagule dispersal by sheep (A) and anemochorous dispersal (B) of *X. cylindraceum* and *X. annuum* seeds compared to a set of alien plant species (see Online Resource 2). Alien species were extracted and classified based on the Romanian national checklist of alien plant species (Anastasiu and Negrean 2009).

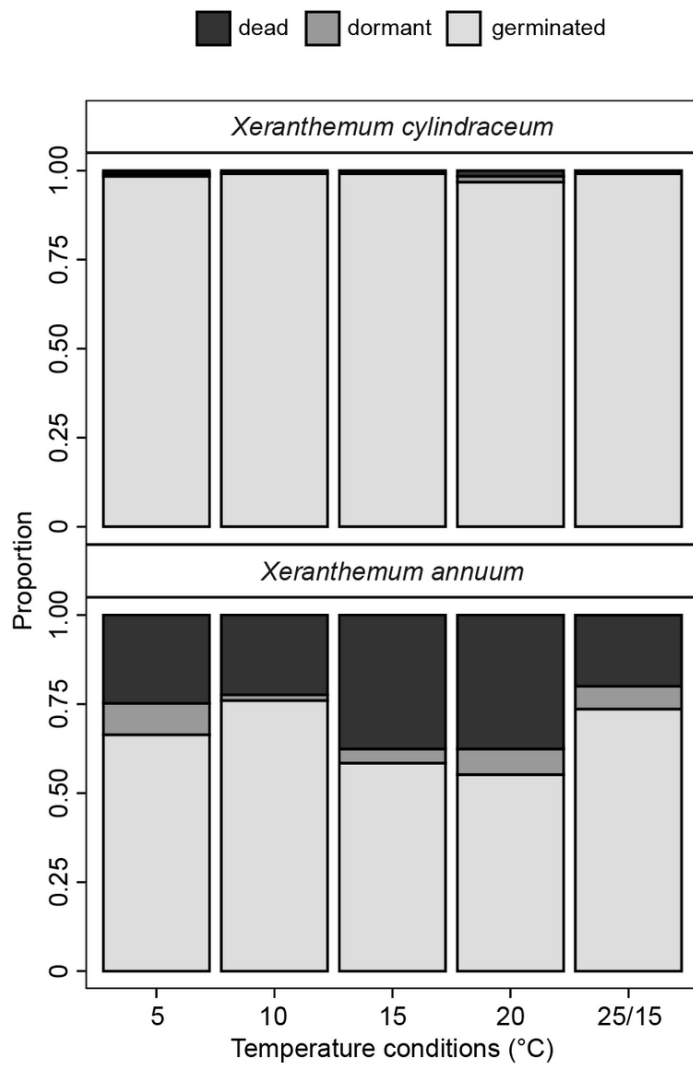


Figure 2

Proportion of germinated, dormant and dead seeds of the two study species at the end of the germination experiment in climate chambers at four constant temperatures and one diurnally fluctuating temperature with a 12 hr light and 12 hr dark photo-cycle.

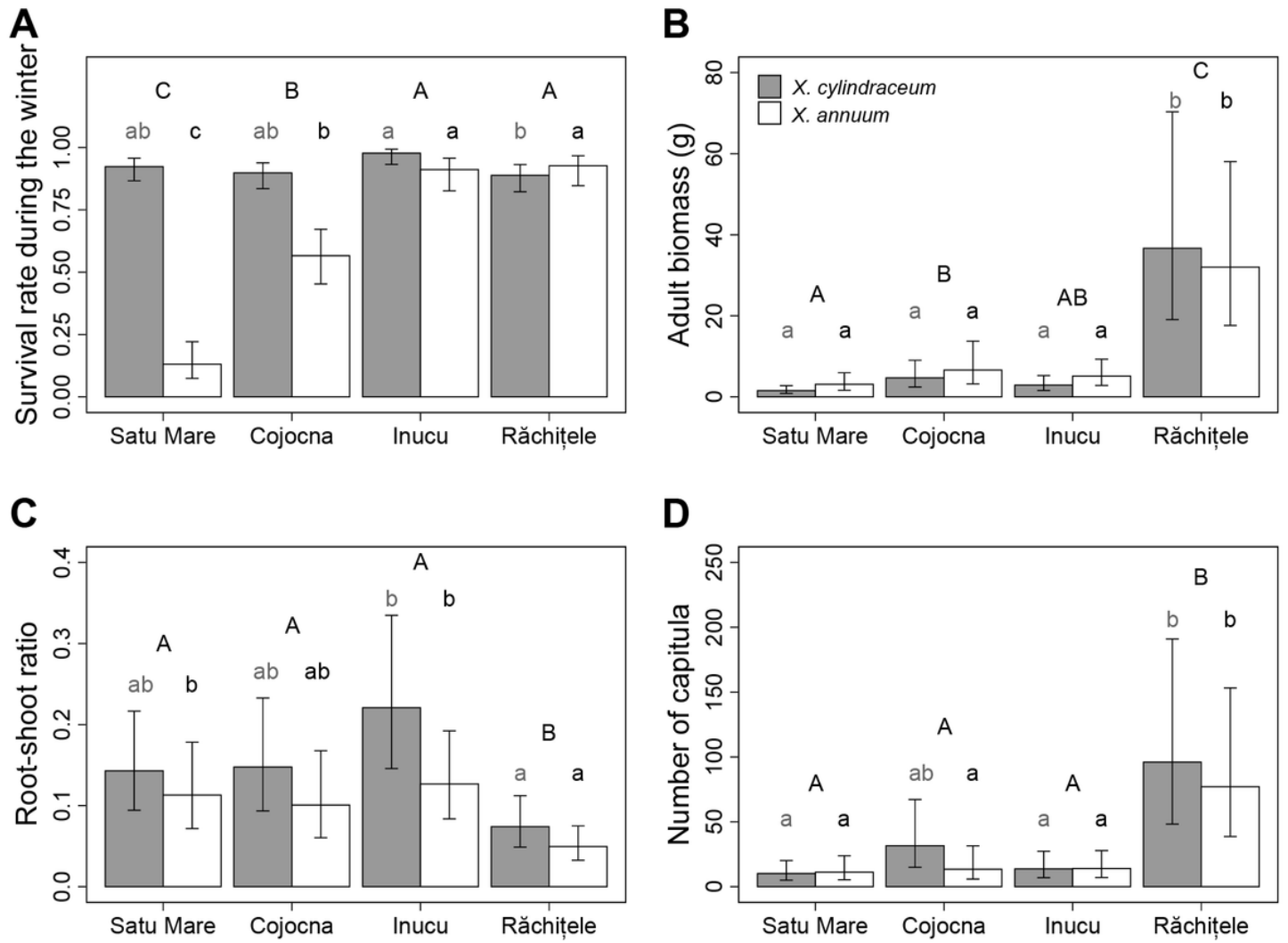


Figure 3

Survival of seedlings during the winter (A), total biomass (B), root-shoot ratio (C) and the number of capitula (bud, flowering, and fruiting stage; D) of *X. cylindraceum* and *X. annuum* specimens at harvest in four transplant sites (for details see Online Resource 1, Table 1). Values are means $\pm$ CI. Letters above the whiskers denote significant differences between the sites and between the two species at $p < 0.05$.

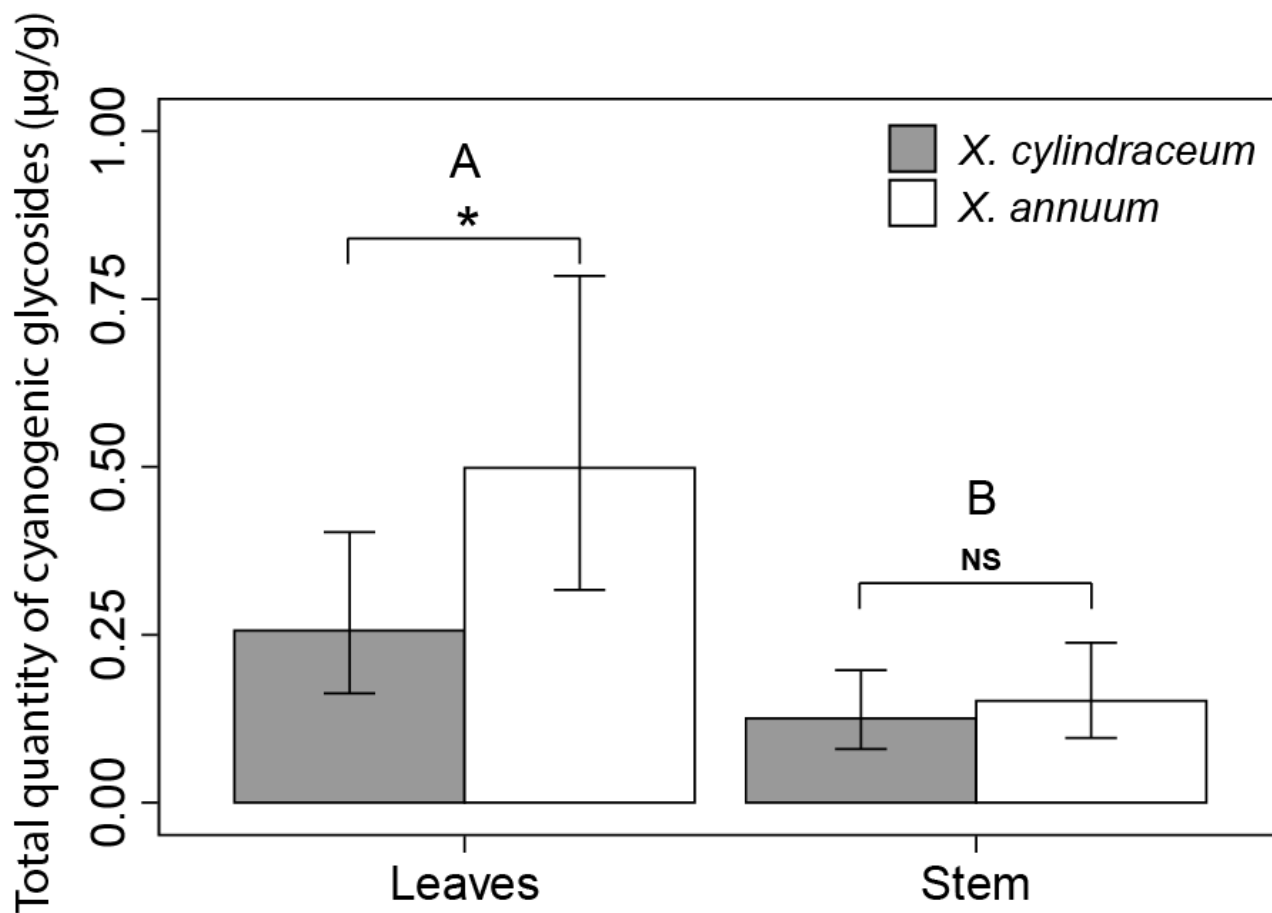


Figure 4

Total quantity of cyanogenic glycosides in leaves and stems of *X. cylindraceum* and *X. annuum*. The result of the ANOVA analysis: the effect of species (SS = 1.09, F = 3.86, p = 0.063), plant parts (SS = 5.45, F = 19.26, p < 0.001) and the interaction term (SS = 0.34, F = 1.22, p = 0.283). Letters above the whiskers denote significant differences between plant parts at p < 0.05, while asterisk denote significant difference between the two species at p < 0.05.

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