

## Research Article

## Reproduction of *Crassula helmsii* by seed in western Europe

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### Abstract

The amphibious plant species *Crassula helmsii* is a widely established and still-spreading alien in various parts of Europe, where it is considered invasive as its dense swards stress the viability of local biota. The species was considered to exclusively reproduce through vegetative means, until *ex situ* germination was recorded from a single locality in Belgium. We assessed whether this seed viability holds on a wider scale, by testing 16 populations from The Netherlands, Belgium, northern France, eastern England and northern Germany in a greenhouse germination experiment. Seedlings were observed from all populations but two, and from each of the five countries. Although most fruits were lacking seeds and the inferred germination percentages were overall low, germinable seed numbers are considerable given the high density of flowering stems. An *in situ* test revealed seeds to make it through normal winter conditions without signs of physical damage and with retention of germinability. Our results suggest that reproduction by seed is a relatively cryptic but widespread phenomenon throughout western Europe. The persistency of seed banks requires further investigation. Nonetheless, these findings already challenge the efficacy of techniques currently applied in *C. helmsii* control.

**Key words:** invasive alien species, aquatic weeds, Australian swamp stonecrop, New Zealand pygmyweed

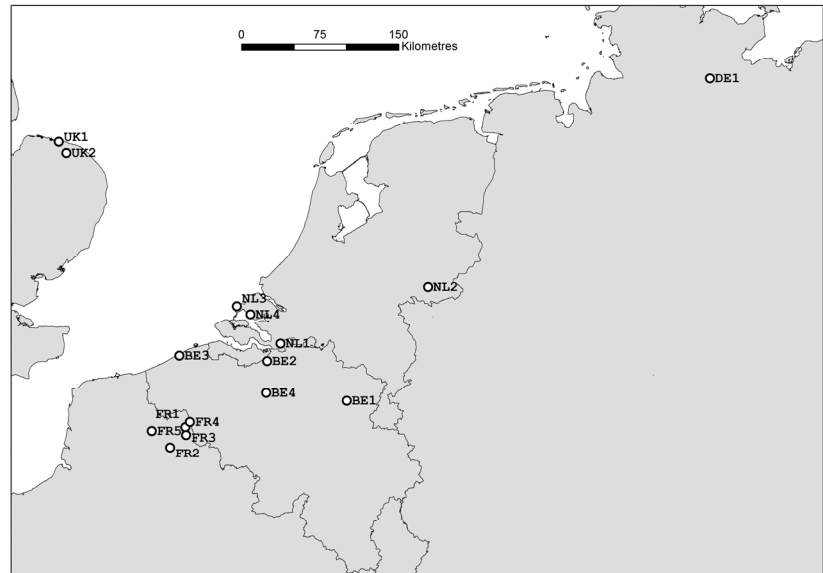
### Introduction

*Crassula helmsii* (Kirk) Cockayne (Crassulaceae) is an amphibious, succulent perennial native to temperate Australia and New Zealand (Webb et al. 1988). The species was introduced into Europe primarily as an aquarium and garden pond plant from the early 20<sup>th</sup> century on (Dawson 1994). The first European records from the wild stem from the second half of the century, and the number of locations has increased very rapidly since. As of 2015, its alien range includes about ten countries in Europe, as well as locations in Russia and in the USA (ISSG 2015; CABI 2015).

*C. helmsii* rapidly forms extensive, monospecific swards in numerous western European wetlands, which is in part facilitated by the species' physiology (Newman and Raven 1995; Klavsen and Maberly 2009, 2010), ecological amplitude (Denys and Packet 2004) and phenotypic plasticity

(Dawson and Warman 1987). Its presence is considered highly problematic for the population viability of local biota (macrophytes, algae, amphibians, invertebrates, even birds; Watson 1999; Langdon et al. 2004; EPPO 2007; Hussner 2009; Martin 2015). Therefore, control measures are widely undertaken. These include manual or mechanical removal (Leach and Dawson 2000), and the application of chemicals (Dawson 1996), hot organic foam (Bridge 2005), nontransparent foils (Wilton-Jones 2005), soluble dyes (Denys et al. 2014) and herbivorous fish (Dawson and Warman 1987). So far, none of these management methods has proven conclusive, with success appearing highly dependent on site conditions (Leach and Dawson 2000).

*C. helmsii* in Europe reproduces readily through vegetative regeneration of turions and stem fragments that include as little as a single node (Dawson and Warman 1987; EPPO 2007). Flowers are small (about 3–3.5 mm in diameter) and are



**Figure 1.** Location of the *Crassula helmsii* populations sampled (for details see supplementary Table S1).

observed in most populations, appearing between July and September (EPPO 2007). Yet, generative reproduction through seed remained particularly enigmatic. Although seed formation was sometimes observed, germination appeared to be absent (Dawson 1994; Delbart 2011). Seed viability was only recently confirmed for the first time in plants that were collected from a single Belgian population and were put under greenhouse conditions (Denys et al. 2014).

This marked observation obviously raises questions on, firstly, whether this latter population is an exception to the rule or not, and secondly, on the fate of seeds under field conditions. We therefore set out to examine the reproductive ability of *C. helmsii* populations from across its western European range, by assessing (1) whether seeds are present, (2) whether these are germinable, and (3) whether they can survive until the next growing season.

## Methods

Plant material for this study was sampled during the fall of 2014 from 16 wild populations in The Netherlands, Belgium, northern France, eastern England and northern Germany (Figure 1, supplementary Table S1). Each sample consisted of several handfuls of uprooted stems from randomly selected patches of flowering plants, which were stored in plastic or paper bags in a dark cold-storage room until further processing.

### *Fecundity measures*

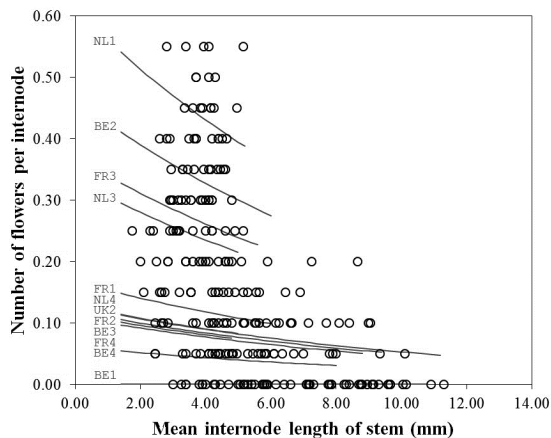
We randomly selected 20 stems per population to determine basic morphological measures. For each stem, we counted the number of flowers that were present on the main axis' 20 most apical internodes. Since stem dimensions and biomass in *Crassula* are known to correlate considerably with level of submergence (Dawson and Warman 1987), we also determined the mean internode length for these stems. This parameter was used as a (co)variate in correlation analysis (Spearman rank correlation) and regression (Poisson regression with population and internode length as independent variables).

In addition, the number of well-developed seeds within fruits was determined by sampling 20 wilted flowers per population (we refer to the four carpels in a flower jointly as a single fruit; Webb et al. 1988).

### *Greenhouse germination*

As *C. helmsii* seeds are minute (< 500  $\mu\text{m}$ ) and were expected to be rare, isolating sufficient seeds for the germination experiment would have proven extremely time-demanding. With the aim of processing a considerable number of populations, we therefore opted to perform such an experiment by 'sowing' fruits instead.

Following stratification (69 to 105 days at 5  $^{\circ}\text{C}$ ), 100 hand-picked, wilted flowers were buried very superficially in sterile sand in plastic trays



**Figure 2.** Relation between mean internode length and the number of flowers in *Crassula helmsii* (20 most apical internodes, 240 stems). The lines are regression curves by population.

(17×13×4 cm, perforated at the bottom). The trays were subjected to a 14h light / 10h dark regime at 18 °C and 12 °C, respectively, and watered by hand using tap water. Up to five such replicates were installed per population (supplementary Table S2). For population BE3, we also included material collected from 2013. The trays were monitored regularly for emerging seedlings for 128 days following installation.

Estimates of germination percentages can only be made indirectly, through the counts of seeds per fruit (if available). Population differences are analyzed by means of pairwise comparisons following a logistic regression model with population as the single explanatory factor (Tukey test at  $p = 0.05$ , Piepho 2012).

Only nine flowers could be isolated from the German sample, with too little material remaining for other measurements. These fruits were put in petri dishes on moistened filter paper instead of trays with sand.

### Winter survival

To determine the fate of seeds under temperate winter conditions in the field, we set up an experiment at the location of BE3, using locally collected flowers and seeds. Either 50 flowers or 10 seeds, with eight replicates each, were fixed in plastic slide mounts between gauze with a 35  $\mu\text{m}$  mesh width (Rasmussen et al. 1993). In December 2014, the mounts were vertically inserted into the soil at randomly located, artificial gaps within the resident *C. helmsii* population. On-site temperature was tracked using a logger installed at about 15 cm above ground level.

Half of the mounts were exhumed in early spring as to determine winter survival (March 2015). The other half were exhumed in late spring as this accords with the optimal germination time for many species of the local flora (June 2015). The mounts were stored in a refrigerator for a single night before being opened. Flowers and seeds were visually checked with a stereoscopic microscope using photographs from before their burial as a reference. The seeds were dissected as to evaluate the condition of the embryos. The flowers were added as replicates to the greenhouse germination experiment (above).

## Results

### Fecundity measures

Mean internode length ranged from 1.75 to 11.30 mm, agreeing well with the documented range and reflecting the species' phenotypic plasticity in response to water level (Dawson and Warman 1987). The number of flowers per stem ranged from zero up to one flower every two nodes (supplementary Table S2). Taking together all the samples, there was a negative correlation between internode length and flower number, indicating that compact plants tended to be the ones flowering most vigorously (Spearman rank correlation coefficient  $r_s = -0.568$ ,  $p < 0.01$ ; Figure 2). However, when applying the Poisson regression model, the variation was significantly represented by population, but only marginally by the stem internode length ( $p = 0.07$ ).

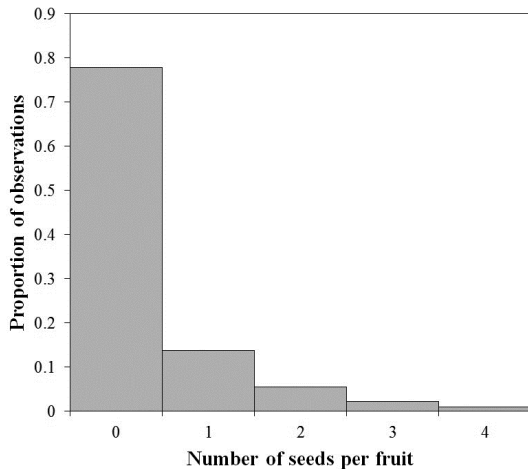
The majority of flowers contained no developed seeds. At most, four seeds per fruit were found (Figure 3).

### Greenhouse germination

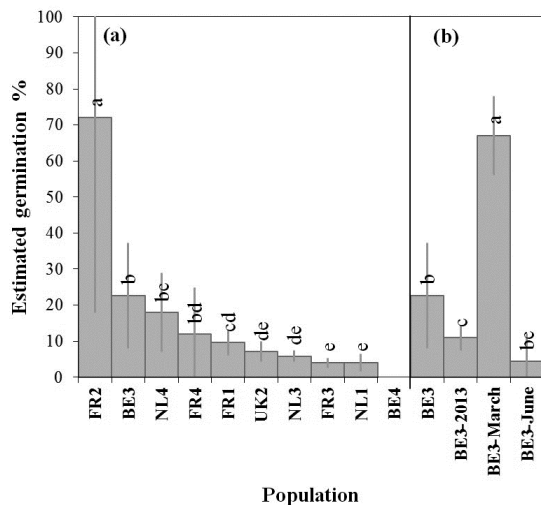
We obtained 222 seedlings from the total of 7009 fruits included in the experiment (supplementary Table S2). Germination occurred from all populations but two (BE2 and BE4), and from each of the five countries. Some seedlings of *Juncus* L. also emerged from the trays, their seeds apparently having hitchhiked along with *C. helmsii* flowers (populations FR5 and UK2).

The first and last seedlings were found at days 13 and 105 following installation, respectively. Emergence was rather irregular, without a clear mode in timing.

Germination was generally low (< 25%; supplementary Table S2, Figure 4). The higher proportion for population FR2 is probably an artifact, given the low estimate of seeds involved.



**Figure 3.** Number of seeds found in fruits of *Crassula helmsii* (240 flowers).



**Figure 4.** **a** Estimated germination percentages (mean  $\pm$  standard deviation) of 10 *Crassula helmsii* populations from across western Europe. **b** Estimated germination percentages for the 2014, 2013, and overwintering samples of the BE3 population. In each panel, populations that share a letter are not significantly different from each other.

### Winter survival

The winter temperature at the study site is shown in Figure 5. Temperatures below 0 °C were recorded on 19 days. The site was inundated from early February until early May (Figure 5). Monthly precipitation and mean temperatures for January–June were normal according to Belgian meteorological standards, except for precipitation in January, which was aberrantly high (Malcorps 2015).

Nearly all seeds were retrieved from the slide mounts exhumed in March 2015 (39 out of 40). All, but one, appeared in perfect condition, though their seed coats had turned markedly darker. From the mounts exhumed in June, 30 out of the 39 retrieved seeds appeared intact, the others showing minor to major ruptures in the coat. One seed had germinated inside the frame.

The flowers, too, were easily retrieved at both occasions, and their perianth appeared only moderately withered. Germination was observed from both batches, with the inferred germination percentage of the batch from March proving relatively high (supplementary Table S2, Figure 4).

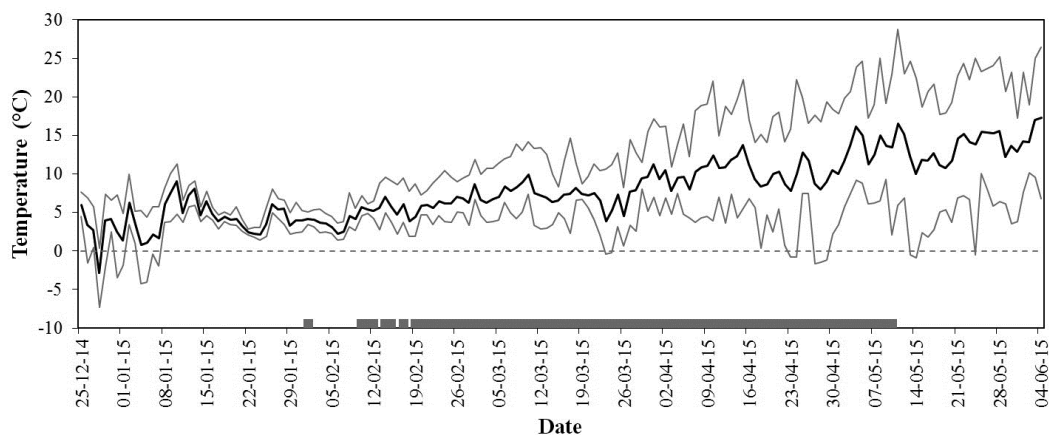
### Discussion

Our results indicate that, contrary to former knowledge, established populations of *C. helmsii* across western Europe have a widespread potential to reproduce generatively by seed.

Actual germination in the field may be very cryptic and hard to quantify when considering the difficulties of identifying seedlings. Indeed, distinction with plants of vegetative origin can only be based on the presence of seed coat fragments, the primary root or the cotyledons, all of which are very small, ephemeral and indistinctive (Dawson 1994). Germination was already suggested by the resurgence of *C. helmsii* after sod-cutting trials or foil application (Adriaens et al. 2010). Yet, the single seedling observed in the slide mounts provides the first documented evidence of *in situ* germination for this part of the species' range.

Although we did not examine the precise conditions necessary for germination, it seems that light and moisture requirements have to be accurately met. Seeds in our experiment were only superficially covered by sand, or at the surface, and we expect this to facilitate germination greatly (Denys et al. 2014). We assume that the irregular germination pattern through time was mainly a consequence of ambient moisture conditions from the water regime applied. Controlling these factors may unravel germination requirements in further detail.

In terms of fecundity, turf-like mats tended to be the ones with most flowers. Yet, the regression model indicated that growth form alone does not predict flower abundance well, but that other population-level factors (either genetic, environmental, or both) must contribute. Note, however, that the link between growth form and fecundity has not been fully explored, as the sampling was biased



**Figure 5.** The daily minimum, mean and maximum temperatures at the BE3 site during the course of the field experiment (lower, middle and upper black lines, respectively). The grey bar at the bottom indicates site inundation.

towards flowering plants in function of the germination experiment. In any case, only a minor fraction of the ovules had turned into seeds, and the explanatory factors remain unclear here, too. Importantly, the species' dense growth largely compensates for the low number of seeds per plant and their limited germination. For population BE3 at 100% cover, we counted an average stem density of 393 stems per dm<sup>2</sup> (min. = 353, max. = 482, n = 6). Assuming that only the 30 youngest internodes have flowers, our numbers suggest an average annual seed production of about 16.000 seeds per m<sup>2</sup>. When taking all the 'worst' parameters together, the estimate yields about 700.000 seeds per m<sup>2</sup>, about a fifth of which would be germinable.

There are some reported observations of *C. helmsii* germinating from sediment samples in its native range, indicating the formation of seed banks (Nicol et al. 2003; Nicol and Ward 2010). However, the longer-term viability of seeds remains unresolved, and it is thus unclear to what extent such banks persist. Short-term persistency seems probable. Indeed, dormancy is a well-known trait in Crassulaceae (Baskin and Baskin 2001), and the terrestrial *C. tillaea* Lester-Garland, for instance, is known to form short-term persistent seed banks (Levassor et al. 1990; Thompson et al. 1997). *C. helmsii* seeds stored for about a one-year period proved to germinate relatively well in our experiment (supplementary Table S2, Figure 4).

Nonetheless, it is already clear that the formation of germinable seeds has crucial implications for *C. helmsii* control. Firstly, they add a new suite

of dispersal means to the plant, as seeds are smaller, lighter, more resistant and less conspicuous than plant fragments (Thiede and Eggl 2007). Secondly, the efficacy of control techniques aimed at plant removal, whether manual, mechanical, chemical or biological, is considerably challenged. Indeed, should persistent seeds prevail, it no longer suffices to eliminate all above-ground vegetation. Further research should therefore look into the formation and dynamics of natural seed banks. This could inform rapid response measures for the prevention of new banks to build up, and may lead to methods for the depletion of existing ones.

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The following supplementary material is available for this article:

**Table S1.** Overview of the stations sampled for *Crassula helmsii*.

**Table S2.** Results of the measurements and the calculations based on them.

This material is available as part of online article from:

[http://www.aquaticinvasions.net/2016/Supplements/AI\\_2016\\_Dhondt\\_et\\_al\\_Supplement.xls](http://www.aquaticinvasions.net/2016/Supplements/AI_2016_Dhondt_et_al_Supplement.xls)