Sexual reproduction of common juniper (*Juniperus communis*) in the face of global change

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De generatieve voortplanting van jeneverbes (*Juniperus communis*) in een globaal veranderend milieu

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Front: *Juniperus communis* seedlings in the germination experiment on the Mechelse Heide

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Woord vooraf

Ik zou liegen moest ik schrijven dat ik al mijn leven lang gefascineerd ben door de jeneverbes. Ook ik dacht vroeger dat dat die rode besjes (aalbessen) waren die weelderig in de Vlaamse moestuinen bloeiden. Ik heb de struik pas leren kennen tijdens mijn studies, en de fascinatie is er pas echt gekomen toen ik op vraag van Kris aan het Labo voor Bos & Natuur mocht werken aan een jeneverbesproject.

De jeneverbes spreekt al eeuwen tot de verbeelding, werd allerlei geneeskrachtige en magische eigenschappen toebedacht en hoorde bij het heidelandschap. Het is een taaie soort die op zeer onherbergzame plaatsen kan voorkomen en erin geslaagd is bijna het volledig noordelijk halfrond te koloniseren. Een overlever ook: als de vermoedens kloppen, is de jeneverbes tijdens de laatste ijstijden niet "teruggetrokken" naar de zogenaamde refugia achter de Alpen en de Pyreneeën, maar is ze, verspreid over Europa, blijven verder groeien.

Fascinatie voor een soort is echter niet genoeg om een doctoraat tot een goed einde te brengen. Tal van mensen wil ik hier graag bedanken.

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En, last but not least, mijn geweldige familie en vrienden! Want er is ook een leven naast een doctoraat!

Content

Summary

With a distribution range that covers most of the northern hemisphere, common juniper (*Juniperus communis* L.) has one of the largest distribution ranges of all plant species on earth. The last decades, however, multiple studies are reporting a significantly declining size and plummeting number of common juniper populations in many regions, including in the western European lowlands and the Mediterranean mountain regions. Failing recruitment is one of the main reasons for this decline. In many of the remaining European juniper populations, the percentage of viable seeds is extremely low, thereby limiting the chances for successful regeneration. However, also other reasons, such as the absence of microclimate suitable for germination and seedling establishment, can be responsible for the lack of recruitment. It was suggested that climate warming as well as enhanced atmospheric depositions can negatively affect juniper's seed viability. However, the mechanisms behind these effects remain unclear. The aim of this thesis was to further our understanding of the effects of climate change (changing temperature and precipitation) and air pollution (atmospheric depositions of nitrogen and sulphur) on different phases of the sexual reproductive phase of common juniper.

We focussed on three crucial seed phases: (seed phase 1, SP1) the early gametophyte development and pollination; (seed phase 2, SP2) further development of the gametophytes, fertilization and early embryo development; (seed phase 3, SP3) late embryo development. The seed viability of populations with and without recruitment was compared. Only after SP3, we found significant differences in seed viability between populations with and populations without recruitment. Hence, this made us conclude that late embryo development is the most crucial phase during seed development. However, anomalies during SP2 can have a delayed effect on embryo development. Therefore, both seed phases were retained for further research.

With both observational and experimental research we studied the effects of global environmental changes on seed viability. Overall, we found viability of SP2- and SP3-seeds to decrease with temperature. Enhanced atmospheric depositions were only negatively correlated with SP3-seed viability.

There are different pathways through which these global-change drivers possibly elaborate their negative effects. For example, increasing temperatures can negatively affect the

development and growth of the female gamethophyte and the pollen tube, the male-female synchrony in the pre-fertilization phases, the development of the megagametophyte and the nutrition and growth of the embryo. On the other hand, both atmospheric depositions and warming can cause nutrient imbalances.

Although the ripening of the embryo (SP3) was identified as the most vulnerable phase, we found strong indications that the actual reasons for low seed viability should be searched in the preceding processes. For example, our transplant experiment along a latitudinal gradient clearly showed that significant differences in seed viability between the temperature treatments only occurred if the seeds also experienced higher temperatures during SP2. Hence, it is plausible that anomalies, induced by warming or enhanced atmospheric deposition, occur during the development of the female gametophyte (SP2). However, their effects only manifested themselves in SP3, for example, by leading to a malfunctioning megagametophyte that will not be able to nourish the developing embryo.

In addition, with a germination experiment, we assessed ideal microclimatic soil conditions for germination and seedling establishment. We found that it is highly important that juniper seeds are in contact with mineral soil, with a sufficient amount of loam or organic matter, for successful germination. For example, sod cutting can create these conditions.

To conclude, our findings highlight that common juniper, arguably one of the most iconic threatened tree species of Europe, is becoming a victim of global change. Successful recruitment in common juniper will only occur if the following conditions are met: enough viable seed must be available (e.g. during a colder period of several years) and enough microsites suitable for establishment must be present. The probability that microsites exist where these conditions (viable seeds and chances for establishment) occur at the same time increases in larger areas where the management is already suited for common juniper. Concerning the low seed viability, attention should be focused on locations where the microclimate is expected to be colder (e.g. northerly oriented slopes or the vicinity of small rivers) and where atmospheric depositions are lower (e.g. not too close to busy roads and/or farms).

This work is also an extra call for policy makers to increase their efforts in reducing the emissions of greenhouse gases and reactive nitrogen.

Samenvatting

Met een verspreidingsgebied dat het grootste deel van het noordelijk halfrond omvat, is jeneverbes (*Juniperus communis* L.) één van de meest wijd verspreide van alle plantensoorten. In verschillende Europese regio's, zoals de West-Europese laagvlakte en de mediterrane berggebieden gaan de laatste decennia echter steeds meer jeneverbespopulaties dramatisch achteruit. Een falende verjonging is een van de belangrijkste redenen voor deze achteruitgang. In veel van de resterende Europese jeneverbespopulaties is het percentage levensvatbare zaden extreem laag, wat de kansen op succesvolle verjonging hypothekeert. Ook andere redenen, zoals het ontbreken van een microklimaat dat geschikt is voor zaadkieming en de vestiging van kiemplanten, kunnen verantwoordelijk zijn voor het uitblijven van verjonging. Er zijn sterke aanwijzingen dat zowel klimaatopwarming als verhoogde atmosferische deposities de zaadvitaliteit negatief beïnvloeden, maar de mechanismen achter deze invloeden blijven onduidelijk. Het doel van deze dissertatie was het verder uitdiepen van ons begrip betreffende de effecten van klimaatverandering (veranderende temperaturen en neerslag) en luchtvervuiling (atmosferische deposities van stikstof en zwavel) op de verschillende ontwikkelingsfasen tijdens de generatieve reproductie van jeneverbes.

Initieel lag de focus op drie cruciale fases tijdens de zaadontwikkeling: (zaadfase 1, SP1) de vroege ontwikkeling van de gametofieten en de bestuiving; (zaadfase 2, SP2) de verdere ontwikkeling van de gametofieten, bevruchting en de vroege embryo-ontwikkeling; (zaadfase 3, SP3) late embryo-ontwikkeling. We vergeleken de zaadvitaliteit tussen populaties met en zonder verjonging en vonden enkel na SP3 significante verschillen in zaadvitaliteit tussen beide groepen. Embryo-ontwikkeling blijkt dus de meest cruciale fase gedurende de zaadontwikkeling. Het is echter mogelijk dat afwijkingen die gedurende SP2 optreden een uitgesteld effect hebben op de embryo-ontwikkeling. Daarom werden beide fases weerhouden voor verder onderzoek.

Met zowel observationeel en experimenteel onderzoek bestudeerden we de effecten van *global change* op de zaadvitaliteit. De zaadvitaliteit van zowel SP2- als SP3-zaden nam af met stijgende temperatuur. Verhoogde atmosferische deposities waren enkel negatief gecorreleerd met de vitaliteit van SP3-zaden.

Temperatuur en atmosferische deposities kunnen op verschillende manieren hun negatieve effecten teweegbrengen. Zo kan een verhoogde temperatuur de ontwikkeling en groeisnelheid van de mannelijke en vrouwelijke gameten negatief beïnvloeden en de ontwikkeling van de megagametofiet en de voeding en groei van het embryo verstoren. Anderzijds kunnen zowel atmosferische deposities als verhoogde temperaturen de nutriëntenhuishouding verstoren.

Hoewel de embryo-ontwikkeling (SP3) werd geïdentificeerd als de meest kwetsbare fase, vonden we sterke aanwijzingen dat de werkelijke redenen voor de lage zaadvitaliteit moeten gezocht worden in de voorgaande processen. Het transplantatie-experiment langs een latitudinale gradiënt toonde duidelijk aan dat een verhoogde temperatuur enkel een negatieve invloed had op de SP3-zaden als deze zaden zich ook al tijdens de voorgaande fase (SP2) bij hogere temperatuurregimes ontwikkeld hadden. Daarom is het aannemelijk dat er al anomalieën ontstaan tijdens de ontwikkeling van de gameten (SP2). Hun effecten worden echter pas zichtbaar tijdens SP3, bijvoorbeeld omdat dit leidt tot een slecht functionerende megagametofiet, die er op zijn beurt niet in slaagt het ontwikkelende embryo te voeden.

Met een kiemexperiment onderzochten we welk beheer tot optimale bodemcondities en geschikt microklimaat voor kieming en vestiging van verjonging leidt. De aanwezigheid van naakte, minerale grond die een zekere vochthoudende capaciteit had bleek hierbij belangrijk. Plaggen is een maatregel die zulke omstandigheden kan creëren.

Onze bevindingen benadrukken dat jeneverbes, misschien wel een van de meest iconische bedreigde boomsoorten van Europa, ook een slachtoffer van *global change* aan het worden is.

Succesvolle verjonging van jeneverbes zal enkel voorkomen als aan verschillende voorwaarden is voldaan: er moet voldoende vitaal zaad aanwezig zijn (bv. na een relatief koude periode van enkele jaren) en er moeten voldoende microsites beschikbaar zijn waar de vestiging van verjonging mogelijk is. De mogelijkheid dat dergelijke omstandigheden zich tegelijk voordoen neemt toe naarmate er grotere gebieden voor handen zijn waar het gevoerde beheer compatibel is met het geschikte beheer voor jeneverbes. Met betrekking tot de lage zaadvitaliteit lijkt het nuttig om te focussen op plaatsen waar kan verwacht worden dat het microklimaat kouder is (bv. op noordelijk gerichte hellingen en in de nabijheid van koude stroompjes) en waar de atmosferische deposities relatief laag zijn (bv. niet te dicht bij drukke wegen en/of boerderijen). Dit werk is ook een extra oproep voor beleidsmakers om hun inspanningen voor het verminderen van de uitstoot van broeikasgassen en reactieve stikstof te verhogen.

List of Abbreviations and definitions

Abbreviations

AIC_c	Akaike's Information Criterion, adjusted for sample size
Al	aluminium
C	carbon
Ca	calcium
$GDD_{>0°C}$	the number of growing degree days above 0 °C base temperature
K	potassium
Mg	magnesium
N	nitrogen
OM	organic matter
OTC	open top chamber
p	significant of statistical test (p-value)
P	phosphorus
S	sulphur
SE	standard error
SD	standard deviation
SP1	seed phase one (cone initiation, pollination, early gamete development)
SP2	seed phase two (gamete development, fertilization and early embryo development)
SP3	seed phase three (late embryo development)
T_{min}	minimum temperature
T_{max}	maximum temperature
VIF	variance inflation factor

Definitions of terms used in this thesis

Archegonium	a multicellular structure in which a single egg is produced
Gametophyte	the haploid, gamete (haploid reproductive cell)- producing generation or phase
Macrospore mother cell	a diploid cell in which meiosis will occur, resulting in the production of four macrospores
Megagametophyte	female gametophyte located within the ovule of seed plants

Nucellus	tissue composing the chief part of the young ovule
Ovule	a structure in seed plants containing the female gametophyte with egg cell, all being surrounded by the nucellus and one or two integuments; when mature, an ovule becomes a seed
Pollen tube	a tube formed after germination of the pollen grain; carries the male gametes into the ovule
suspensor system	structure at the base of the embryo; it pushes the embryo into nutrient rich tissue of the female gametophyte
zygotes	diploid cell resulting from the fusion of male and female gametes

1 Introduction

1.1 Common juniper: a threatened species in many parts of Europe

With a distribution range that covers most of the northern hemisphere (between latitudes of 30° to 70°; Hultén & Fries 1986, Adams 2008), common juniper (*Juniperus communis* L.) is one of most widespread plant species on earth. It occurs in a wide variety of ecosystems (see e.g. Fig. 1-1, Fig. 1-3, Fig. 1-4) from dunes at sea level, heathlands and forests in lowlands, on mountains at elevations of up to 3570 m and in the Arctic tundra. The last decades, however, multiple studies are reporting a declining size and plummeting number of common juniper populations in different regions, including in the northwestern European lowlands (e.g. Belgium: Frankard 2004 and Adriaenssens *et al.* 2006; the Netherlands: Oostermeijer & De Knegt 2004; northern and western Germany: Hüppe 1995; England: Clifton *et al.* 1997), and the Mediterranean mountain regions (García *et al.* 1999). Although the species can locally still be very abundant and exhibit a good regeneration (e.g. in the Alps, Scandinavia and Poland; Falinski 1980, Rosén 1995, Rosén & Baker 2005), *J. communis* communities are listed in Annex I of the EU Habitat Directive (code 5130) due to their threatened status in several European regions.

Fig. 1-1. Common junipers on calcareous grassland (Sweden)

Numerous studies have aimed to unravel the cause of the decline of common juniper (e.g. García *et al.* 1999, Verheyen *et al.* 2005, Ward 2007, Verheyen *et al.* 2009, Zeidler *et al.* 2009). Habitat loss and degradation and the limited sexual regeneration in the remaining populations might be important causes (Verheyen *et al.* 2005). While the protected status of *J. communis* communities has largely stopped habitat destruction across Europe and stimulated appropriate management actions such as the removal of competing vegetation, regeneration is still problematic in many of the remaining populations (e.g. García *et al.* 1999, Verheyen *et al.* 2005). More specifically, Verheyen *et al.* (2009) revealed a triangular relationship between the percentage of viable seeds and the regeneration potential in European common juniper populations (Fig. 1-2). This relationship suggests that if seed viability is low, recruitment is negligible, while in case of a high percentage of viable seeds other factors such as herbivory, summer drought and the absence of suitable microsites for germination are responsible for the differences in recruitment between populations.

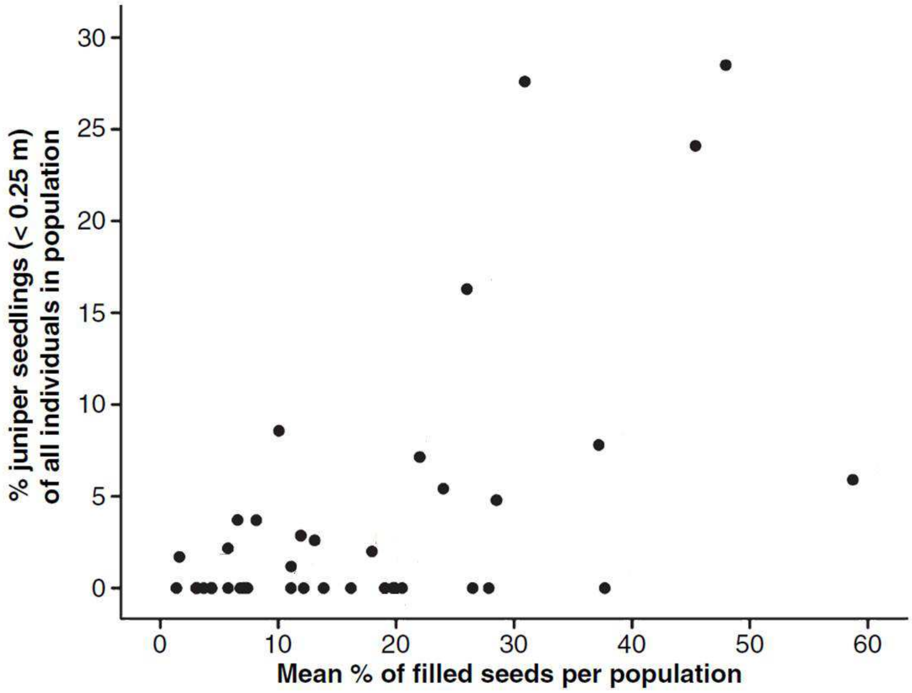


Fig. 1-2. Relationship between the mean percentage of filled seeds per shrub and the percentage of juniper seedlings (i.e. shrubs < 25 cm) in 39 populations distributed throughout Europe (Verheyen *et al.* 2009).

In many of the remaining European juniper populations, the percentage of viable seeds is extremely low thereby limiting the chances for successful regeneration (García 2001; Verheyen *et al.* 2009). In populations where viable seeds are present, other factors such as the absence of microsites suitable for germination, overgrazing and drought stress most likely hamper successful recruitment (Ward 1973, Fitter & Jennings 1975, Gilbert 1980, Ward 1982, García 2001). It remains unclear why some juniper populations have high seed viability and seem to recruit successfully while others exhibit extremely low seed quality. However, there are strong indications that different global change drivers are, at least partly, responsible for the bad reproduction in common juniper (Verheyen *et al.* 2009). Additionally, even on the same shrub, some juniper seeds have a two year ripening time and some seeds have a three year ripening time (Ward 2010). It is not clear what causes this dual strategy and whether ripening time has an influence on seed viability.

1.2 Global change

Worldwide, environmental conditions and ecosystems are undergoing rapid change (MEA, 2005). The drivers of this global change have mostly anthropogenic causes, and can be coupled in five groups: climate change, land-use change, pollution, overexploitation and biotic invasions. They all influence plant species and populations (MEA 2005, Matesanz *et al.* 2010). For example, land-use change, leading to habitat loss, fragmentation, reductions in habitat quality and habitat degradation (Matesanz *et al.* 2010), caused a general decrease of heathlands (an important habitat for common juniper, see Chapter 2) throughout Europe in the last two centuries (e.g. Piessens *et al.* 2004, Alonso 2004, Newton *et al.* 2009). However, given the protected status of many juniper populations in western Europe and the findings of Verheyen *et al.* (2009) concerning the negative effects of temperature and nitrogen deposition on seed viability, we focus in this thesis on the global change drivers of climate change and pollution.

Climate change

In the Northern hemisphere, the average surface temperature has increased with 1.0 °C since the end of the 19th century (NASA GISTEMP data, http://data.giss.nasa.gov/gistemp/). This warming is unparalleled in the last millennia (Marcott *et al.* 2013). The present climate change is almost exclusively caused by anthropogenic factors. Human-induced changes in the world's radiation balance amount to 2.29 Watt per m² since 1750, while natural causes due to changes in solar radiation contribute only 0.06 Watt per m² (IPCC 2013). Hence, only climate models in which anthropogenic causes are accounted for are capable of reconstructing the observed temperature trends. In a 'business-as-usual'-scenario (RCP8.5), a worldwide temperature increase of 2.6 to 4.8 °C is expected by the end of the 21st century (2081-2100) compared to the average between 1986 and 2005 (IPCC 2013). Models forecast strong climate-change impacts on biodiversity with a reduction of more than 50 % of the current climate range for 57 % of the plants and 34 % of the animals during the 21st century (Warren *et al.* 2013).

Pollution

Before industrialization occurred in the 19th century, natural processes largely dominated nitrogen emissions (~120 TG N/yr) due to low anthropogenic inputs (~12 Tg N/yr). By 2005, the input by anthropogenic processes spectacularly increased to ~210 Tg N/yr (Galloway *et al.* 2004). Hence, it is no surprise that worldwide atmospheric deposition of biologically reactive N more than tripled from 1860 to the early 1990s. Moreover, by the year 2050 a 2.4 to 2.7-fold increase in eutrophication of terrestrial ecosystems, compared to 2000, is predicted, probably causing an unprecedented biodiversity loss and subsequent biotic homogenisation of ecosystems (Tilman *et al.* 2001). This decades-long N deposition is partly responsible for the already significant losses of terrestrial plant diversity (Clark & Tilman 2008; De Schrijver *et al.* 2011). Nitrogen emissions include emissions of NO_y (NO, NO_2, HNO_2, HNO_3 and NO^-_3), which mainly originates from burning of fossil fuels, and of NH_x (NH_3 and NH_4^+), mainly emitted by agricultural practices. Apart from the eutrophying effects on ecosystems (Bobbink *et al.* 2010), N-depositions can also cause an acidifying effect. Potentially acidifying depositions comprises deposition of SO_x (both SO_2 and SO_4^{2-}), NO_y and NH_x compounds, and can cause a significant acidification of the soil by by generating protons (H^+). Although the emissions of sulphur (S) have been reduced over much of Europe in the past decades (UN-ECE 2003), nitrogen emissions to the atmosphere remain elevated in industrialized regions and are accelerating in many developing regions (Galloway *et al.* 2004). Hence, soil mediated acidification is still a problem in many ecosystems (Bobbink *et al.* 2010).

Fig. 1-3. Common junipers in heathland (Belgium)

The effects of global change will become increasingly visible in the coming decades (Rands *et al.* 2010) and further the change of environments in which plants are living. The early life-history stages of plants, including seed production, are amongst the most important processes that drive plant community structure (Hedhly *et al.* 2009, HilleRisLambers *et al.* 2009, Linkies *et al.* 2010, Walck *et al.* 2011). Sexual reproduction aids plants to adapt to changing environments and to colonize previously unoccupied habitats (Fenner and Thompson 2005). Hence, in the face of global change, the role of sexual reproduction in the adaptation of organisms to changing environments might become more important. However, sexual reproduction itself is also affected by global change. Several studies have investigated the influence of different global-change drivers on sexual reproduction of plants, including warming (Peñuelas *et al.* 2004, De Frenne *et al.* 2011, Koivuranta *et al.* 2012), elevated CO_2 concentrations (Thurig *et al.* 2003), nitrogen deposition (Callahan *et al.* 2008) and drought (Demirtas *et al.* 2010). Fewer studies have investigated the integrated effects of several drivers on sexual reproduction (Hovenden *et al.* 2008, HilleRisLambers *et al.* 2009, Verheyen *et al.* 2009, Li *et al.* 2011). Even less is known about the different processes acting during subsequent phases of the sexual reproductive cycle of plants (but see Owens *et al.* 2001, Hedhly 2011). Recently, Hedhly *et al.* (2009) underlined the importance of studying the sensitive phases (e.g. fertilization, embryogenesis) independently in order to obtain a better understanding of the effect of global change drivers (i.e. temperature) on sexual reproduction.

1.3 Objectives and outline of this thesis

The aim of this thesis is to get a better understanding of the problematic sexual reproduction of common juniper. Before focussing on sexual reproduction, we first give a short introduction to the species in Chapter 2. Next, we provide an in-depth study of global-change effects on seed development (Chapters 3, 4, 5 and 6). In addition, as only a part of the recruitment problem is caused by low seed viability (Verheyen *et al.* 2009), we also studied the establishment process (Chapter 7). In Chapter 8, we synthesize our findings and add some recommendations for common juniper conservation.

Fig. 1-4. A common juniper ticket in Zutendaal (Belgium)

Fig. 1-5 gives a schematic overview of the outline of this thesis. Three observational studies (Chapter 3-5) were performed to unravel the influence of climate warming and atmospheric depositions on seed viability. Following the recommendations of Hedhly *et al.* (2009), we first searched which phases (1, cone initiation and pollination; 2, gamete development, fertilization and early embryo development; 3, late embryo development) during sexual reproduction were most vulnerable (**Chapter 3**). In this chapter, seeds were sampled in eight populations across Europe (four with recruitment and four without recruitment). Next, we studied the influence of climate warming and atmospheric depositions on the two most vulnerable phases (phase two and three, i.e. gamete development, fertilization and early embryo development, and late embryo development) in **Chapter 4**. For this research, we sampled seeds and studied their viability in not less than 42 populations throughout Europe and took advantage of the large variation in both temperature and atmospheric depositions. In a subset of twenty populations, we also analysed the chemical composition of the needles to verify whether the nutrient status of the shrubs was influenced by climate warming and atmospheric depositions and whether this might be one of the mechanisms by which they exert their negative influence on seed viability (**Chapter 5**). The experiment in **Chapter 6** was used to corroborate the observational findings using the gradient method in Chapter 4 concerning the negative influence of increasing temperature on seed viability. Here, we installed a transplant experiment at four locations in Europe, along a temperature gradient. At one of the four locations we also performed an open top chamber (OTC) experiment. In this experiment we increased the temperature in one of the two plots at that location by placing a glasshouse with open top above the shrubs.

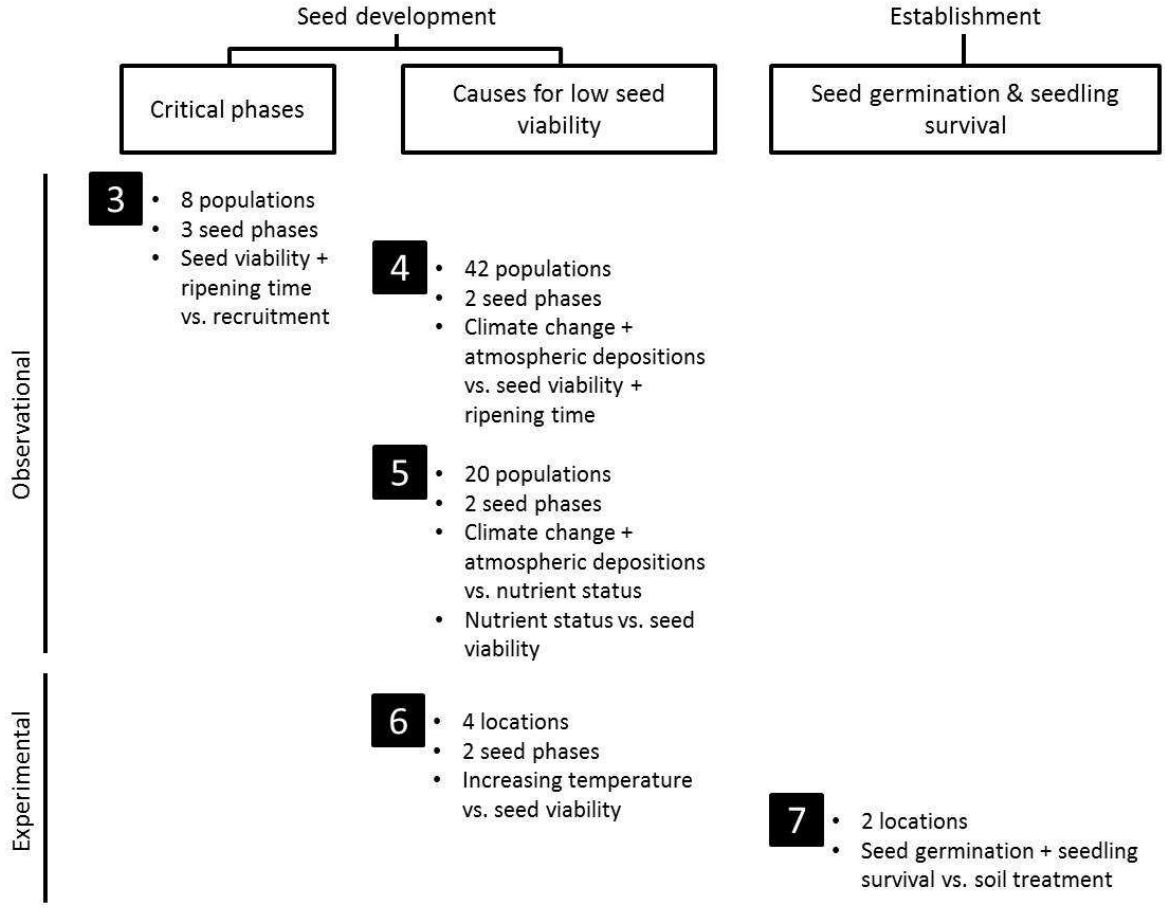


Fig. 1-5. Schematic overview of the outline of this thesis. Chapter numbers in black frames.

Finally, **Chapter 7** focusses on the establishment of common juniper seedlings. In an experiment performed at four locations in Belgium and the Netherlands, the effects of different management actions such as mowing or sod removal on the soil conditions were observed. Subsequently, we studied how these management actions and soil conditions affected seed germination and seedling survival.

2 Common juniper: a short introduction

2.1 Botanical characteristics

Juniperus communis (Cupressaceae) is a light demanding, dioecious, wind pollinated, coniferous shrub or tree (Thomas *et al.* 2007). The form of the dense crown can be quite variable and three phenotypes can be distinguished (Fig. 2-1). The columnar shape has steeply ascending branches along the main stem. Secondly, the fastigate form has a fairly steep but widening crown without a clear main stem. Finally, the prostate form is a low-to-the-ground shrub with horizontal branches and bending tops. There can be quite some variation within one type (Stockmann 1982). In juniper thickets, the columnar shape is mostly absent, while solitary individuals have often the fastigate form.

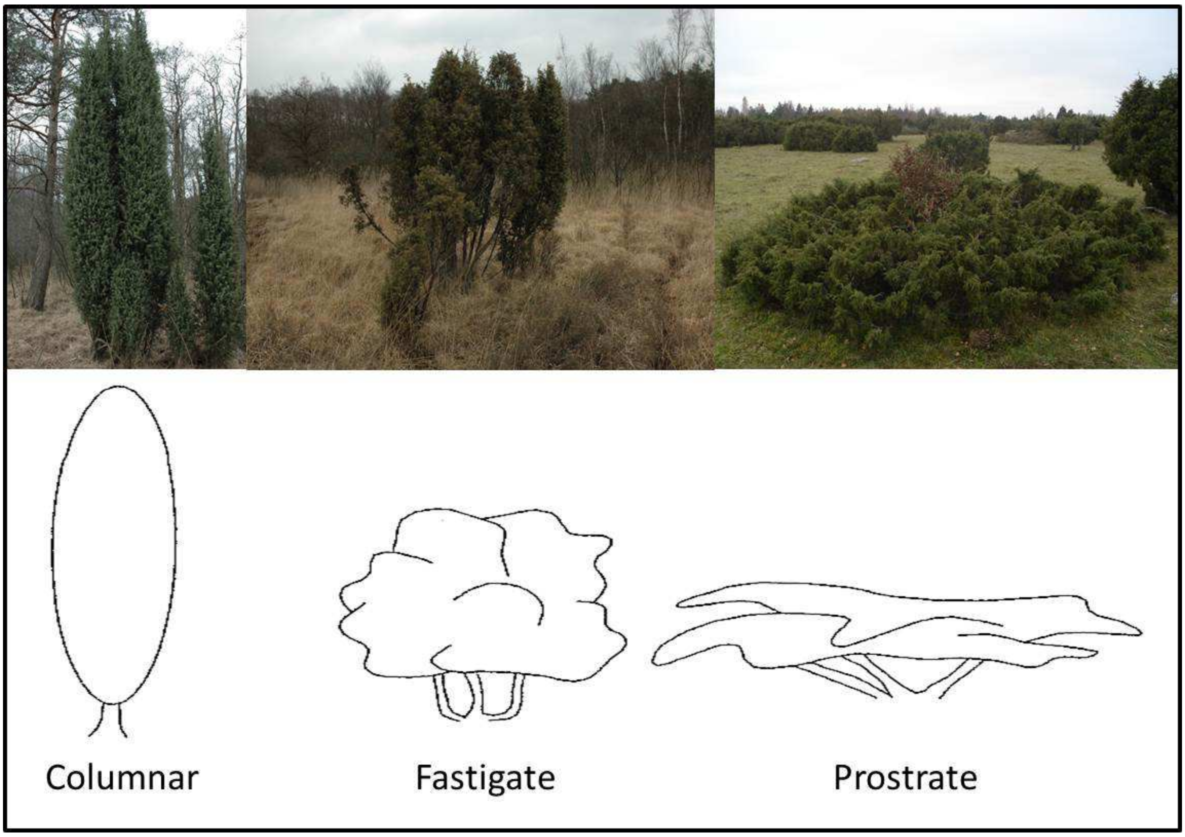


Fig. 2-1. The three growth forms of common juniper (Stockmann 1982).

The leaves are exclusively needle-shaped, prickly, blue-green and about 10 to 15 mm long (Fig. 2-2, Fig. 2-3). The upper side of the needles is dull and trough-shaped, while the underside is shiny and keeled. The needles are placed in whorls of three on hexagonal branches and remain in place for two years (Weeda *et al.* 1985). Due to their high light demands, the needles in the center of the crown quickly die off (Vanhaeren 1983). Common juniper has relatively nutrient rich litter and can enhance soil fertility. For example in alpine heath tundra, patches of common juniper shrubs can facilitate the growth of other plants typical for this ecosystem (DeLuca & Zackrisson 2007).

Fig. 2-2. Needles and female cones of common juniper (Belgium).

Fig. 2-3. Morphological characteristics of common juniper. 1 = male cone, 2+3 = stamen, 4 = female cone, 5 = female cone (longitudinal section), 6 = ripe female cone, 7 = cross-section of cone with three seeds with numerous resin reservoirs, 8+9 = longitudinal section of a seed, A = male twig with cones, B = female twig with cones. Images are not to scale (Thomé 1885).

2.2 Distribution

Of all vascular plants, common juniper has one of the largest distribution ranges. It covers most of the northern hemisphere (Adams 2008) (Fig. 2-4).

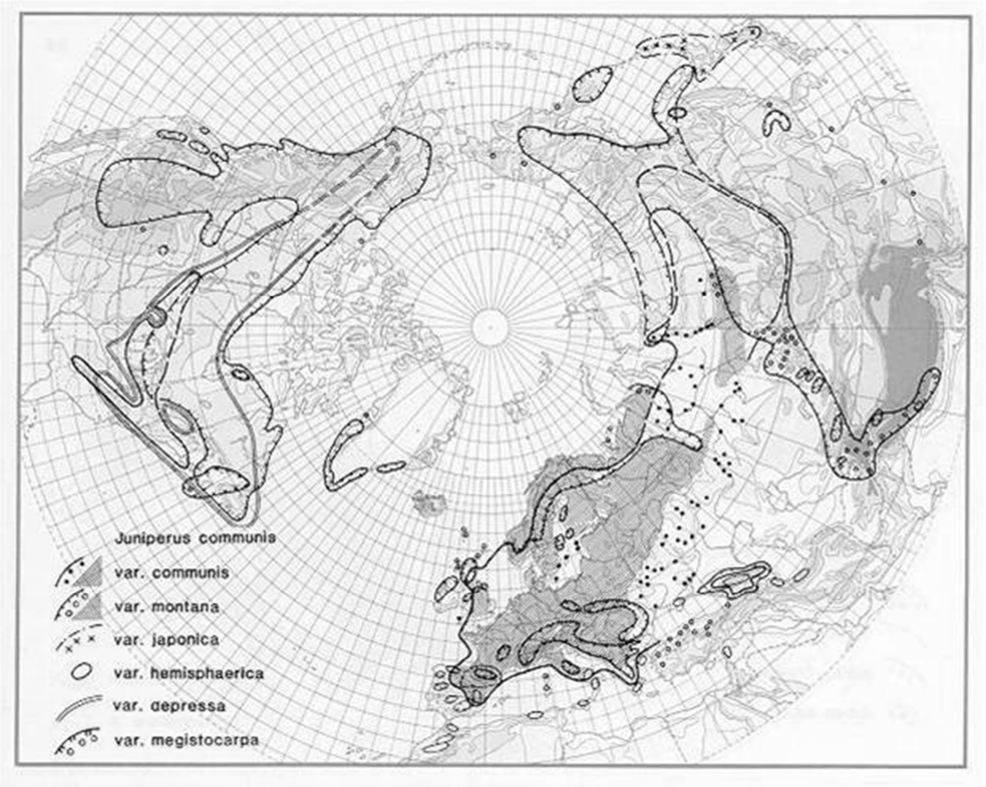


Fig. 2-4. Distribution area of common juniper in the Northern hemisphere (Hultén & Fries 1986). In our research we focussed *on Juniperus communis* var. *communis*.

In Europe, the species occurs in the Atlantic area, in the continental regions and from the boreal climates to the mountains of the Mediterranean area. Outside Europe, common juniper can be found in areas with a temperate climate in northern Asia towards the Tanshian mountains (except in Japan) but also in Iraq and the Himalaya. In America, common juniper occurs from Canada to New Mexico in the USA. In the south, the species mostly occupies higher altitudes. The species is only absent in very warm and dry areas, such as the Mediterranean lowlands.

2.3 Sexual reproduction

The female bushes of juniper annually produce fleshy, spherical, berry-like cones of approximately 6.5 mm in diameter that take two or three years to mature. The cones contain one to three (rarely four) seeds (Ottley 1909, García *et al.* 2000, Thomas *et al.* 2007, Ward 2010). The seed development is described in Fig. 2-5, and is based on Ottley (1909), Singh (1978), Thomas *et al.* (2007) and Ward (2010).

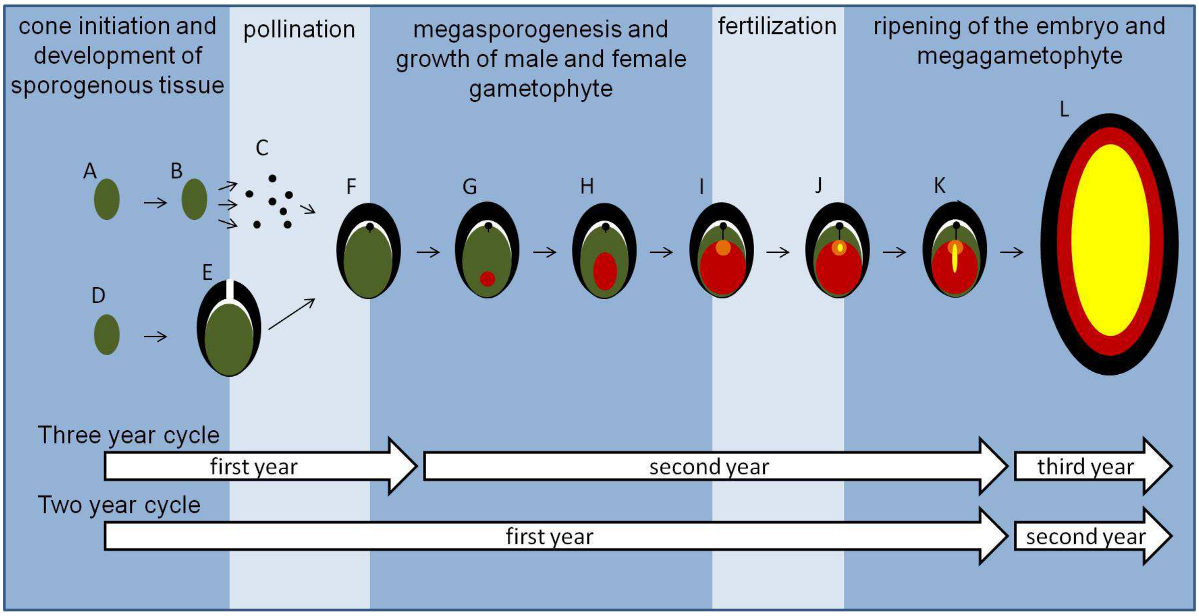


Fig. 2-5. Schematic overview of the seed development in *Juniperus communis*. (A) Male cone containing the microsporangia; (B) the microspores are formed after meiosis; (C) pollen are released; (D) female cone (green) with three ovules; (E) ovule with integument (black) and nucellus (green); (F) after pollination, the pollen (black) germinates on top of the nucellus and the pollentube grows towards the centre of the nucellus, the integument has closed; (G) + (H) in the centre of the nucellus, the macrospore mother cell undergoes meiotic divisions and forms the prothallium (red) which will grow and develop into the megagametophyte; (I) the archegonial complex develops from peripheral cells at the micropilar end of the prothallium (orange) and consists of four archegonia, each containing one egg cell; (J) the pollen tube has reached the archegonial complex and after fertilization one or more zygotes are formed (yellow); (K) several embryonal masses are pushed in the corrosion cavity of the megagametophyte through elongation of the suspensor system (yellow); (L) generally only one embryo (yellow) survives in the mature seed. (redrawn after Ottley 1909, Singh 1978, Thomas *et al.* 2007, Ward 2010)

In a three year cycle the initiation of the reproductive buds starts in autumn or early winter. When ripe, the male strobili have three to seven pair or trio's of sporofyls that all contain two to six sporangia (Chambers *et al.* 1999; see Fig. 2-6 for ripe male cones). Pollination and the germination of the pollen takes place in spring. The megasporogenesis follows next spring and, subsequently, the female gametophyte starts to develop. After fertilization in second summer, the maturation of the embryo and megagametophyte begins and the seed is ready for dispersal by the end of next year's autumn. In a two year cycle, the megasporogenesis and development of the female gametophyte advance with one year and follow immediately after pollination. Fertilization then takes place in the first summer. The female cones reach their full size shortly after fertilization. Their colour changes from green to dark blue/purple during summer and autumn of the last year of development (Ward 2010).

Fig. 2-6. A branch with male common juniper cones (Belgium)

Seeds that are ready for dispersal have a dormant embryo and need to undergo an after ripening-process before they can germinate (Pack 1921). Different treatments have been tested to improve germination (e.g. Pack 1921, McVean 1966, Broome 2003, Adriaenssens *et al.* 2006) with variable success. However, good results in breaking dormancy are achieved if the seeds are stored at a temperature between 0 and 10 °C with an optimum of 4 - 5 °C (Pack 1921, McVean 1966, Broome 2003). For example, Broome (2003) found a germination percentage of 50 to 60 % (viable seeds) if seeds were stored at 0 °C for 34 to 51 weeks with an after-treatment of 16 to 17 weeks at 4 °C. Afther the pre-treatments, seeds were sown in spring and a germination period of three month followed. Pack (1921) found that storage in cold (-23 °C) and moist conditions or in conditions of altering temperatures negatively affected germination. McVean (1966) and Broome (2003) advise to use cleaned seeds, but Adriaenssens *et al.* (2006) mention good germination results if cones are sown.

2.4 Seed dispersal

Gravity, animals and to a lesser extend wind and water disperse the seeds of common juniper (Piotto *et al.* 2003). Most of the cones will land under or in the close proximity of the mother shrub (Knol & Nijhof 2004). Mainly through birds, there is a chance that the seeds get more widely dispersed. Species from the thrush family (*Turdus* spp), such as fieldfare (*T. pilaris*), mistle trush (*T. viscivorus*) and common blackbird (*T. merula*) are often mentioned, but also Eurasion magpie (*Pica pica*), willow tit (*Parus montanus*) and black grouse (*Tetrao tetrix*) occur as dispersers (Bergman 1963, Breek 1978, García 2001). In addition, according to Ward (1973) and Rosén (1988), junipers are often found along sheep walks. Hence, possibly also sheep play an important role in the seed dispersion.

Although birds are often mentioned as important dispersers, less is known about the influence of e.g. frugivory on the seed viability. Pack (1921) supposed that the mechanical handling in the crop and the effects of digestive juices on the seed coat would facilitate the germination of the seed. However he found no effects when he treated the seeds with diluted citric acid. Livinston (1972) reported a strong reduction of germination percentages (from 37.2 % to 9.1 %) after seeds passed through the stomach of starlings (*Sturnus vulgaris*). However, a treatment with citric acid increased the germination percentage afterwards.

2.5 Growth conditions

Common juniper typically occurs on dry, both acidic and calcareous, nutrient poor, mineral soils, such as heath and calcareous grasslands (Weeda *et al.* 1985, García *et al.* 2000, Rosén & Bakker 2005, Maes *et al.* 2006, Thomas 2007, Ward 2007). The species can also survive in open forest, but does not resist heavy shadowing (Thomas *et al.* 2007). Persistently high or fluctuating groundwater levels are detrimental. However, individuals can sporadically be found in moist conditions.

Due to its slow growth (Grubb *et al.* 1999, Ward 2007) and its high light demands (Thomas *et al.* 2007), common juniper often suffers from the competition of other species. It tolerates poor soils (Pearman *et al.* 2008), but will grow relatively faster on richer soils. As common juniper is predominantly appearing on nutrient-poor, both acidic and calcareous soils and harsh environments (García *et al.* 2000, Thomas 2007, Ward 2007) it is plausible that the species is excluded from its optimal habitat (e.g. richer soils) by more competitive species (Pearman *et al.* 2006), that make better use of the available nutrients.

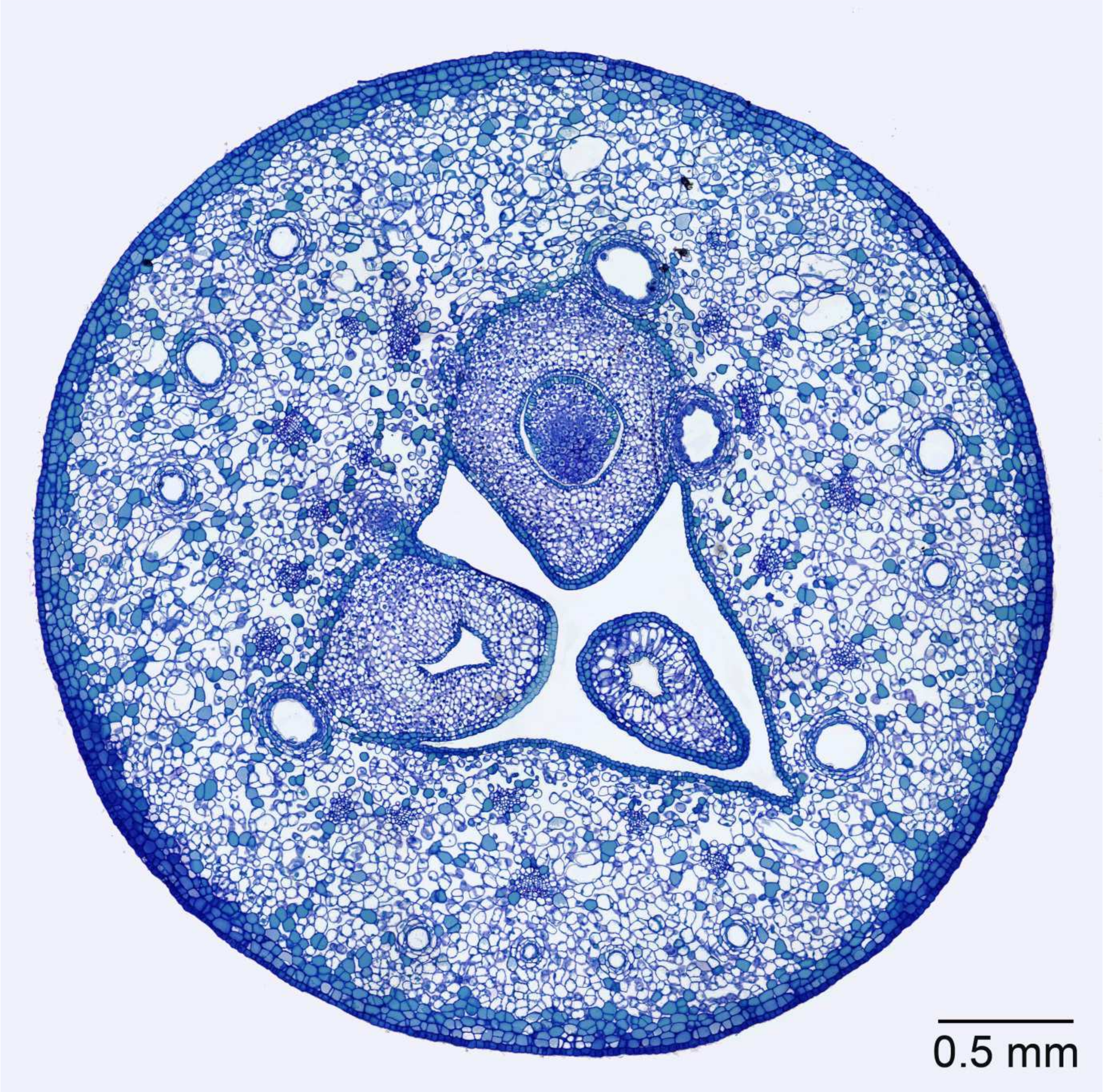
2.6 Human use of common juniper

The cones, needles, branches and wood of common juniper had, and still have, multiple applications. For example, the branches were used as decorations for religious ceremonies and weddings (Burny 1985, Barkman 1989), to flavour bread, as a broom (Knol & Nijhoff 2004) and the wood was used for the production of tools on a small scale (Barkman 1989).

The cones are used in gin and alcoholic bitters. They can flavor marinades, pot roasts, liver pâté, game, sauces and soups (Charles 2013).

Traditionally, common juniper also has a medicinal use. For example, essential oils from the cones showed to be bactericidal and extrasts from the needles and cones have a good antioxidant activity (Charles 2013). As an herbal medicine, common juniper has been used as a steam inhalant against bronchitis (Shahmier et al. 2003).

In addition, extracts of common juniper are applied in cosmetics and perfumery (Shahmier et al. 2003).


0.5 mm

3 Critical phases in the seed development of common juniper (*Juniperus communis*)

After Gruwez R, Leroux O, De Frenne P, Tack W, Viane R, Verheyen K. 2013. Critical phases in the seed development of common juniper (*Juniperus communis*). Plant Biology 15:210–219

Abstract

Common juniper (*Juniperus communis* L.) populations in northwestern European lowlands are currently declining in size and number. An important cause of this decline is the lack of natural regeneration. Low seed viability seems to be one of the main bottlenecks in this process. Previous research revealed a negative relation between seed viability and both temperature and nitrogen deposition. Additionally, the seeds of common juniper have a variable ripening time, which possibly influences the seed viability. However, the underlying mechanisms remain unsolved. In order to unravel this puzzle, it is important to understand in which phases of the seed production the main defects are situated and what the influence of ripening time is. In this study, we compared the seed viability of populations with and without successful recruitment. We examined three seed phases: (1) gamete development; (2) fertilization and early embryo development; (3) late embryo development. After the first two phases we found no difference in the percentage of viable seeds between populations with or without recruitment. After the late embryo development populations without recruitment showed a significant lower percentage of viable seeds. These results suggest that the late embryo development is a bottleneck in the seed development. However, the complex interaction between seed viability and ripening time makes it plausible that the causes should be searched in the second seed phase, as the accelerated development of the male and female gametophyte may disturb the male-female synchrony for successful mating.

3.1 Introduction

Common juniper populations are declining in size and numbers in different regions, including northwestern European lowlands (e.g. Belgium: Frankard 2004 and Adriaenssens *et al.* 2006; the Netherlands: Oostermeijer & De Knegt 2004; northern and western Germany: Hüppe 1995; England: Clifton *et al.* 1997), and the Mediterranean mountain regions (García *et al.* 1999). Limiting sexual reproduction, caused by low seed viability may be one of the main causes for this decline (Verheyen *et al.* 2005, Verheyen *et al.* 2009). Moreover, Verheyen *et al.* (2009) found indications that increasing temperature and nitrogen deposition negatively affect seed viability. However, the mechanisms behind the low seed viability remain largely unclear. Nevertheless, a range of possible causes of seed abortion in conifers has been identified, including a lack of ovule pollination, developmental anomalies, early or late self-incompatibility mechanisms, and insect and disease damage (Owens 2006). In many conifer species, seed abortion occurring in different developmental phases of the seed often leads to the so called “empty seeds”, which generally still contain some remnants of the megagametophyte or aborted embryo (Owens & Morris 1998, Owens 2006, Owens *et al.* 2008). Seed coat development, independent of embryo abortion was also observed in common juniper (García *et al.* 2000).

The following developmental phases can be distinguished: initiation of the reproductive buds, development of the sporogenous tissue, meiosis, pollen dispersal and landing, development of the male and female gametes after pollination, fertilization, the formation of the proembryo, the elongation of the suspensor, the establishment of the polar meristems (root and shoot) and the further development of the embryo (e.g. Singh 1978, Bonner 2008, Hedhly *et al.* 2009). For different conifer species crucial phases were identified by comparing the percentage of viable seeds between the phases mentioned above (Owens 1995, Owens & Morris 1998). Similar research is still lacking for common juniper.

The variation in ripening years of the seeds is another important factor to take into account in common juniper. Normally, common juniper seeds ripen in three years (Thomas *et al.* 2007). However, Ward (2010) found that in different populations the majority of the seeds ripen in two years. She suggests that the three year cycle is more frequent in populations with a colder climate (and vice versa) and that the ripening time can influence the seed vitality in some phases.

In order to achieve a better comprehension of the mechanisms behind the low seed viability in *J. communis* in many European populations, we compare the seed viability in different seed development phases between populations with and populations without recruitment (i.e. the presence of shrubs smaller than 0.5 m) across northwestern Europe. In addition, we assessed the possible link between seed viability and ripening time for the different seed development phases.

3.2 Methods

3.2.1 Sampling, population characteristics and climate data

We selected eight large common juniper populations across northwestern Europe: four with recruitment (Ekulunde in southern Sweden, Kleszczele in eastern Poland, Rossdach in central Germany and Ascholdinger in southern Germany) and four without recruitment (Mantinge and Markelo in the Netherlands and Zutendaal and As in Belgium) (Fig. 3-1).

Fig. 3-1. Location of the eight sampled populations in northwestern Europe (the black symbols mark the populations without recruitment, the grey symbols those with recruitment).

Bushes smaller than 0.5 m were considered as recruitment and were frequent in the first four populations. The canopy cover above the common junipers was lower than 20%. Climate data of each population were obtained from the NewLocClim 1.10 software (F.A.O. 2005), using nearest-neighbour interpolation of ten weather stations. We deduced mean annual temperature and mean temperature of the warmest and coldest month for the period 1961–1990 for all populations (Table 3-1).

In the autumn of 2008, we randomly located a 30 × 30 m plot in each population (except in Ekulunde, where the quadrant was 50 × 50 m). In each plot, the height of all the *J. communis* bushes was recorded in six height classes ($\leq$ 0.5m, $\leq$ 1 m, $\leq$ 2 m, $\leq$ 3 m, $\leq$ 4 m, > 4 m) and, in addition, per plot, seven to eight female, cone bearing bushes were selected at random. On each selected individual, we randomly sampled ten cones of one, ten cones of two and ten cones of three years old. The age of the cones was deduced from the age of the wood on which they were growing (the cones are one year younger than the wood), which, in turn was determined by counting the growing shoots using the annual bud scars, bud scales and comparative leaf lengths (leaves are short and brownish near the annual scar) (Ward 2010). The cones were stored in 70% ethanol.

Table 3-1. Coordinates of the eight sampled populations with their mean annual temperature, the mean temperature of the coldest and warmest month, the density of adult juniper bushes (i.e. bushes >0.5m) and the density of juniper recruitment (i.e. bushes ≤ 0.5m).

Population	Latitude (°)	Longitude (°)	Altitude (m.a.s.l.)	Mean annual temperature (°C)	Mean temperature coldest month (°C)	Mean temperature warmest month (°C)	Adult indivuals (ha^{-1})	Indivuals ≤0.5m (ha^{-1})
Ekulunde	56. 6	16.6	45	6.56	-2.10	15.80	288	604
Kleszczele	52.6	23.3	100	6.63	-4.90	17.20	2556	1322
Rossdach	50.0	11.1	440	8.35	-1.00	17.70	333	111
Ascholdinger	47.9	11.5	550	7.87	-1.80	17.50	322	1378
Zutendaal	50.9	5.6	80	9.44	2.00	17.20	167	0
As	51.0	5.6	75	9.44	2.00	17.20	622	0
Markelo	52.3	6.5	50	8.94	1.60	16.30	478	0
Mantinge	52.8	6.6	50	8.58	1.20	16.00	489	0

3.2.2 Seed morphology and anatomy

Cone colour was observed and the occurrence of sessile sucking scales (*Carulaspis* spp., Homoptera, Diaspididae) (García 1998) was checked for each of the sampled cones. Based on visual inspection of the cones we determined three phases in their development (cone phases): (1) small green cones, hardly distinguishable from leaf buds; (2) large green and blue-green cones, the size being approximately the size of ripe cones; (3) large, blue ripe cones, ready for dispersal.

As the seeds were sampled in late autumn, we expected to distinguish three different phases in the seed development (Fig. 2-5): the pollinated ovule with a germinating pollen on the nucellus (seed phase one, further referred to as SP1; Fig. 2-5F), the seed with the suspensor system pushing the embryonal masses in the corrosion cavity (seed phase two, further referred as SP2; Fig. 2-5K) and the mature seed, ready for dispersal (seed phase three, further referred to as SP3; Fig. 2-5L).

In order to characterize the three observed seed phases and the corresponding criteria for viable seeds, ten seeds from each seed phase were studied in detail. The seeds were pulled out of the cones and transverse sections were made with a rotation microtome after embedding the seeds in 7100 Technovit, following Leroux *et al.* (2007). The coupes were examined under a light microscope. The results of this study are reported in chapter 3.3.2.

The quantitative aspect of our analysis was based on stereoscopical observation of dissected seeds. Our detailed anatomical study serves (see 3.3.2) as a base for interpretation and classification. Seeds in any of the three phases (SP1, SP2 and SP3) that had no visible signs of anomalies were considered to have the potential to develop to the next phase and are further referred to as “viable seeds”. We also reported if the anomalies were caused by the seed predator chalcid *Megastigmus bipunctatus* (Hymenoptera, Torymidae; see Roques & Skrzypczynska (2003) for a review of the seed-infesting chalcids of the genus *Juniperus*) or by mites (e.g. *Trisetacus quadrisetus* (Acarina, Eriophyiidae)).

Based on the seed phase and the age of the cone we calculated the ripening time of the seeds (an SP2 seed of one year old or an SP3 seed of two years old has a ripening time of two years, an SP2 seed of two years old or an SP3 seed of three years old has a ripening time of three years) and two groups were distinguished: (1) seeds that ripen in less than three years and (2) seeds that ripen in three or more years.

3.2.3 Data analysis

Firstly, we examined the correlations between seed viability, the age of the seed, the cone phase and the seed phase with a non parametric Spearman's rank correlation test. The test was performed for every population separately.

To analyse the differences in seed viability between populations with and without recruitment generalized linear mixed-effect models were applied using the *lmer* function in the *lme4* library in R 2.13.0 (R Development Core Team 2012). As viability of the SP1, SP2 and SP3 seeds, ripening time and infection with *M. bipunctatus*, mites and *Carulaspis* spp. were scored as 0 or 1, a binomial distribution was used (for ripening time, seeds that had a ripening time of three or more years were scored 1, seeds that had a ripening time of less than three years were scored 0). To analyse the viability of the SP1, SP2 and SP3 seeds, population, bush and cone were included as nested random effect terms. Random effect terms for the analysis of *M. bipunctatus,* mites and *Carulaspis* spp infection and ripening time were population and bush.

To study the relationships between the viability of the SP1, SP2 and SP3 seeds on the one hand and ripening time and infection with *Carulaspis* spp. on the other hand, generalized linear mixed-effect models with binomial distributions were used. Population, bush and cone identity were again included as nested random effect terms. To detect possible multicollinearity between the predictor variables, the variance inflation factors (VIF) were calculated (Quinn & Keough 2002) before running the model. For the three seed phases, VIF's were lower than 10 which indicates relatively low multicollinearity (Quinn & Keough 2002). Seeds infected with mites and *M. bipuncatus* were considered as non-viable and the cones of SP1 seeds never contained sucking scales. Therefore, correlations of these variables with seed viability were not calculated.

3.3 Results

3.3.1 Populations

The two groups of four populations each were selected on the presence or absence of recruitment. Fig. 3-2 shows that not only the frequency of recruitment distinguished the two groups, but also the height distribution showed strong differences.

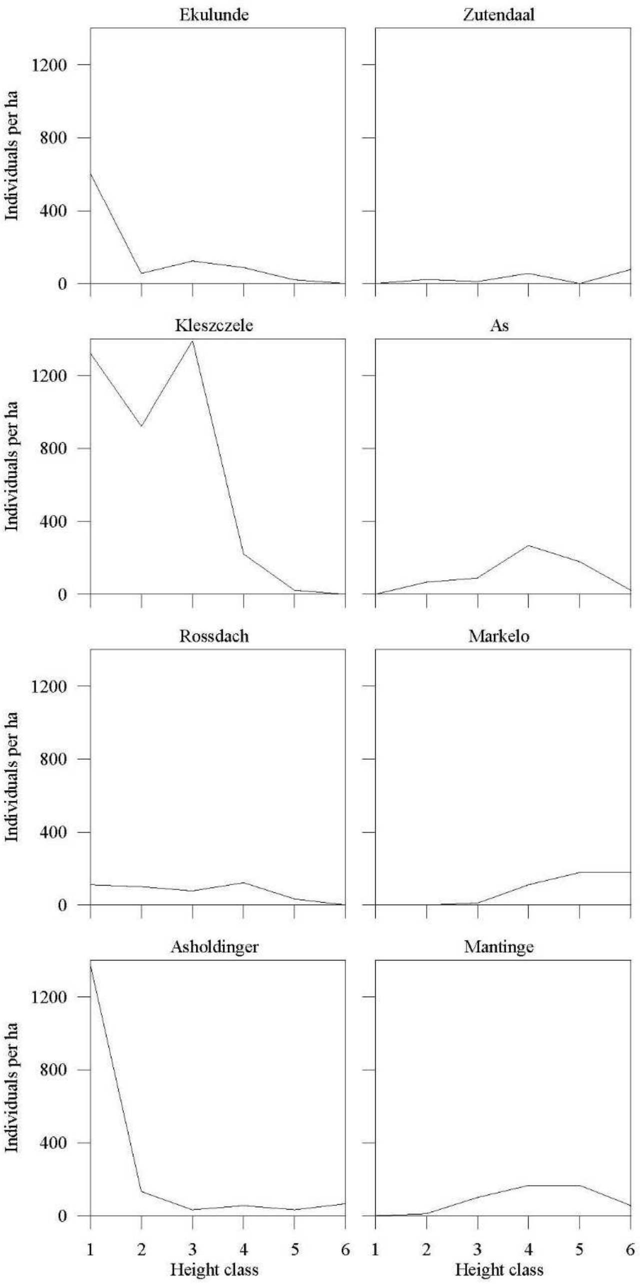


Fig. 3-2. Height distribution of the sampled populations expressed as number of individuals per ha within a particular height class (1: ≤ 0.5m; 2: ≤ 1.0m; 3: ≤ 2.0m; 4: ≤ 3.0m; 5: ≤ 4.0m; 6: > 4.0m). A distinction is made between successfully recruiting (left) and non-recruiting populations (right).

The inverted J-shaped height distribution of the populations in Ekulunde, Kleszczele, and Ascholdinger, and to a lesser degree in Rossdach, demonstrate that they all had relatively more individuals in the lower height classes than in the higher ones. This suggests that conditions for sexual regeneration (e.g. seed viability and suitable microclimate for germination) are better than those in the populations in Mantinge, Markelo, Zutendaal and As.

3.3.2 Morphological and anatomical seed observations

We were able to distinguish three different phases in the seed development (Fig. 3-3). In the first seed phase (SP1), the pollen grain had already germinated and was visible on top of a brown, smooth nucellus (Fig. 2-5F; Fig. 3-3B). SP1 appeared only in the small green cones. Seeds with anomalies such as a shrivelled or black nucellus or the absence of germinating pollen were classified as non viable.

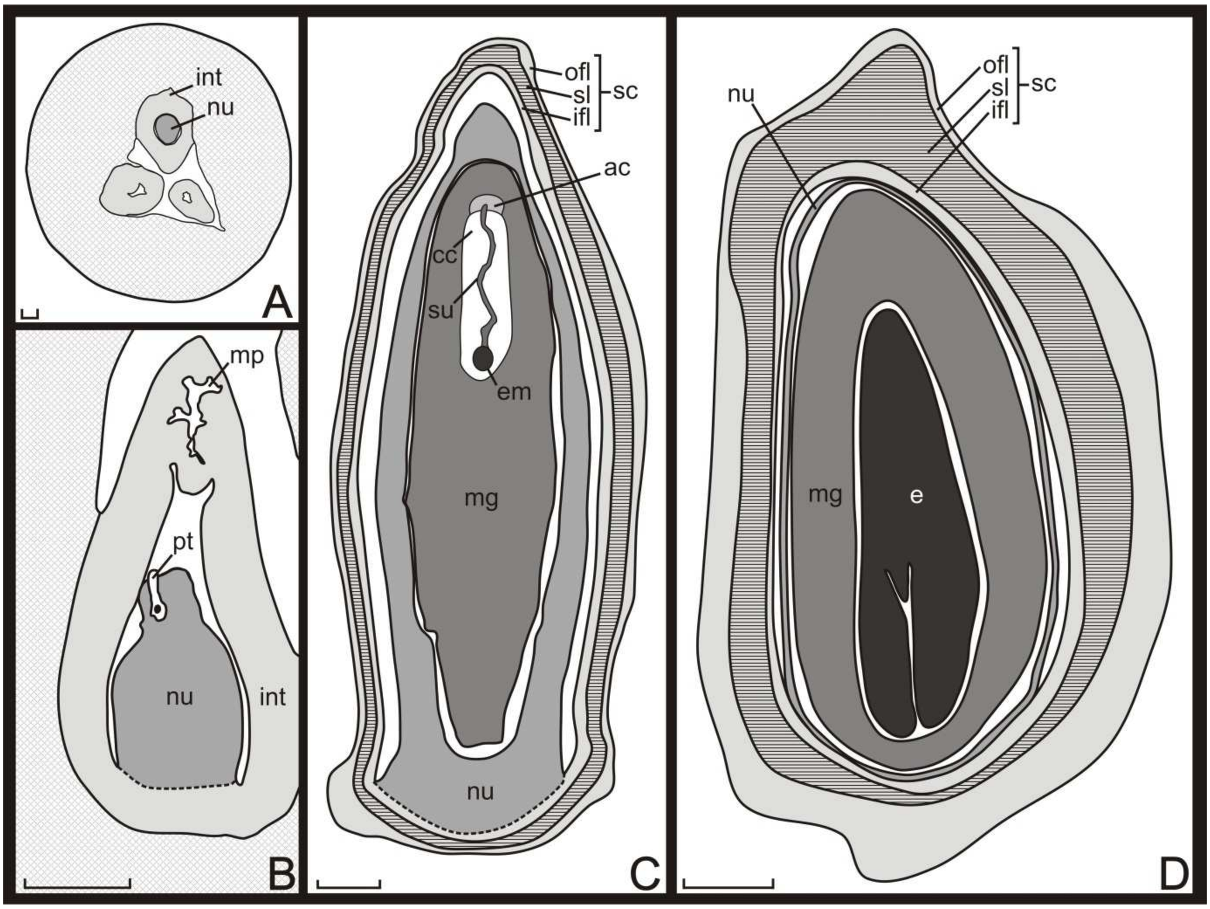


Fig. 3-3. Schematic representations of the three observed seed phases. (A) Transverse section through a cone with three ovules (seeds after SP1) in the centre. (B), (C) and (D) longitudinal sections through seeds after SP1, SP2 and SP3 respectively. (int) integument; (nu) nucellus; (pt) pollen tube; (cc) corrosion cavity; (ac) archegonial complex; (su) suspensor system; (em) embryonal mass; (mg) megagametophyte; (mp) micropyle; (sc) seedcoat, with inner fleshy layer (ifl), sclerified layer (sl) and outer fleshy layer (ofl); (e) embryo. Scale bars: 200 µm.

In seed phase two, most of the seeds that were present in the large green cones showed evidence of early embryo development (Fig. 2-5K; Fig. 3-3C). The state of development of the seeds was characterized by the presence of the suspensor system and different clumps of embryonal mass in the corrosion cavity (Fig. 3-3C, only one embryonal mass is represented).

The megagametophyte was surrounded by the remnants of the nucellus and had not filled all the available space within the seed coat. Both megagametophyte and nucellus consisted of green-white and moist tissue. Failures during the period of fertilization mostly lead to abortion of the megagametophytes, leaving a collapsed, brown sac-like structure, consisting of the megaspore wall. Sometimes, the degeneration was not completed, but the content of the seeds had already dried out. The third seed phase (SP3) that could be distinguished was present at the end of the predispersal embryo development. The embryo and megagametophyte were fully grown and the space within the seed coat was almost completely filled (Fig. 2-5L; Fig. 3-3D). The two cotyledons of the embryo were clearly distinguishable. Both the embryo and the megagametophyte had a smooth, white and moist surface. Anomalies during the embryo development can lead to degeneration of the embryo and/or the megagametophyte, leaving, apart from the brown sac-like structure, some other remnants in the seeds, varying from brown, dry material to a partly developed embryo and/or megagametophyte. In both SP2 and SP3, seeds were occasionally damaged by mites or by *M. bipunctatus*. The content of seeds attacked by mites is completely distorted and mostly, the mites are still present. Damage by *M. bipunctatus* could be recognized by the granular content, an exit hole or the presence of the larva.

Although this method for assessing seed viability (i.e. visually controlling for abnormalities in the seed) generates an overestimation of the real seed viability, Adriaenssens (2006) stated that there is a clear correlation ($R = 0.681$ and $p < 0.01$) with the results of more precise methods such as a tetrazolium test (Miller, 2004). In such a test, a solution of 2,3,5 trifenyl tetrazolium choloride is added to seeds that are hydrated and cut longitudinally. In the presence of living tissue, the colourless solution turns reddish.

3.3.3 Correlations between seed viability, seed age, cone phase and seed phase

As expected, we found a strong positive correlation ($p < 0.01$) between seed age, cone phase and seed phase for every population (Appendix A). The relationship of these three variables to the seed viability was always strongly negative ($p < 0.01$) with the exception of the correlation between seed viability and age in Asholdinger ($p < 0.05$) (Appendix A). Hence, we concluded that the seed phase equals the seed age and cone phase as good proxy for seed viability. Therefore we opted to study seed viability using the three different seed phases.

3.3.4 Differences between populations with and without recruitment

The percentage of viable seeds decreased with each successive seed phase for both populations without (69%, 24% and 3% after SP1, SP2 and SP3 respectively) and with recruitment (49%, 22% and 13% after SP1, SP2 and SP3 respectively; Fig. 3-4).

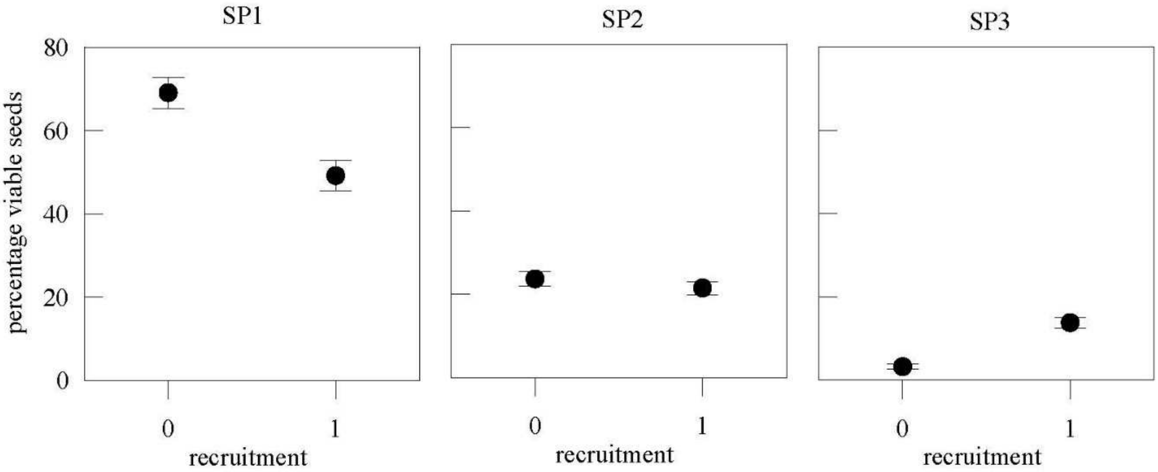


Fig. 3-4. The percentage of viable seeds after seed phase one, two and three (SP1, SP2 and SP3, respectively) in juniper populations with (1) and without (0) successful recruitment across Europe. Error bars denote the standard error of mean.

Populations without recruitment displayed a higher percentage of viable SP1 seeds than populations with recruitment. However, the opposite was true for SP3 seeds. Only for SP3 seeds, the difference between the two groups was statistically significant ($p < 0.001$) (Table 3-2). The proportional decrease of viable seeds peaked in phase SP3 for populations without recruitment: up to 88% of the viable SP2 seeds degenerated during the third seed phase compared to only 36% in populations with recruitment.

Table 3-2. The difference between recruiting and non-recruiting populations for different response variables related to juniper seed viability and plant performance in four populations with and four populations without recruitment (SE stands for Standard Error).

Response	Estimate	SE	z-value	p-value
Seed viability SP1	-1.2925	0.9254	-1.397	n.s.
Seed viability SP2	-0.3148	0.9231	-0.342	n.s.
Seed viability SP3	2.2506	0.6319	3.562	<0.001
Probability of *M. bipunctatus* infection	0.6981	0.6970	1.002	n.s.
Probability of mite infection	-1.715	1.339	-1.281	n.s.
Ripening time	0.9612	1.0569	0.910	n.s.
Probability of infection with *Carulaspis* spp.	-2.1509	0.8714	-2.468	<0.05

Only one of the other variables tested showed significant differences between the two groups (Fig. 3-5; Table 3-2). In populations without recruitment, a higher percentage cones were infected with *Carulaspis* spp. The percentage of seeds infected with *M. bipunctatus* was higher in populations with recruitment, although this was not significant (Table 3-2). The percentage of seeds infected with *M. bipunctatus* or mites was always very low, mostly below 6% (Fig. 3-5).

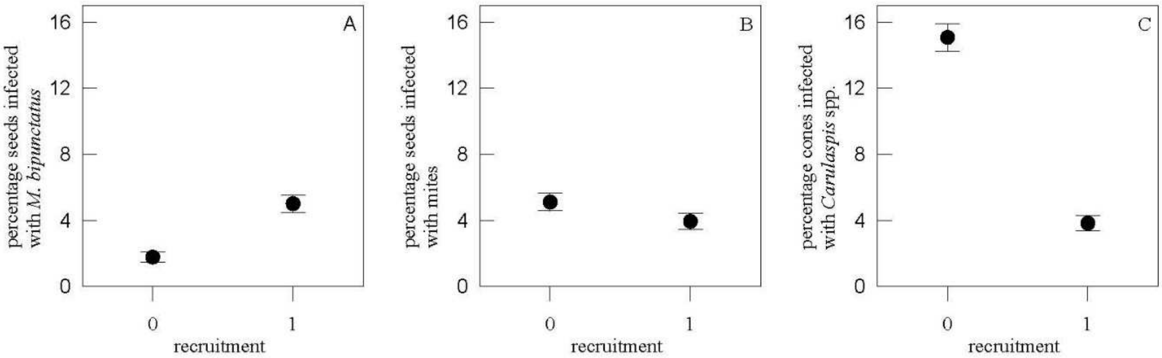


Fig. 3-5. Differences between populations with (1) and without (0) recruitment concerning the percentage of seeds that is infected with *M. bipunctatus* (A) and mites (B) and the percentage of cones that is infected with *Carulaspis* spp. (C). Error bars denote the standard error of mean.

Most of the studied seeds ripened in less than three years (68%). More specifically, only the populations in Ekulunde and Kleszczele had a higher percentage of seeds that ripened in three or more years (Fig. 3-6). Seeds that ripen in three or more years were absent or negligible in the populations in As and Asholdinger. After seed phase three, also populations in Zutendaal and Mantinge showed very low numbers of seeds that needed a longer ripening time.

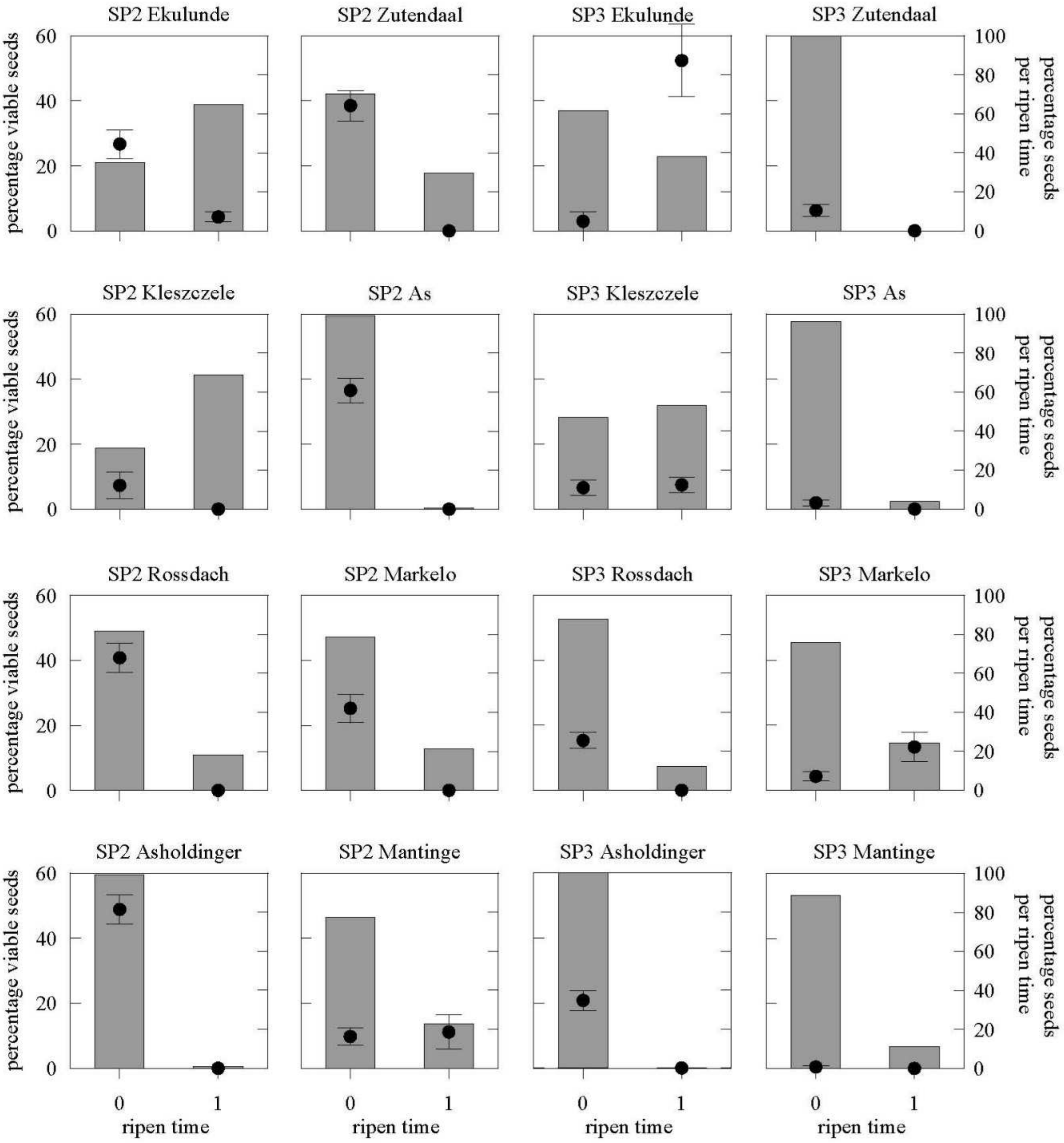


Fig. 3-6. Differences in percentage of viable seeds (black dots, primary Y-axis) between seeds that ripen in less than three years (0) and seeds that ripen in three or more years (1) after seed phase two (SP2) and seed phase three (SP3). Error bars denote the standard error of mean. The grey bars represent the percentage of seeds that ripen in less than three years (0) and in three or more years (1) per population (secondary Y-axis). A distinction is made between recruiting (first and third column) and non-recruiting populations (the second and fourth column) and between seed phase two (first two columns) and seed phase three (last two columns).

3.3.5 Factors explaining seed viability

Surprisingly, the infection probability with *Carulaspis* spp. had no significant influence on the seed viability in spite of the differences between populations with and without recruitment (Table 3-3). The ripening time showed a strong negative correlation with the viability of SP2 seeds: seeds that developed slower had a lower chance to be viable (Table 3-3).

Table 3-3. The effects of ripening time and probability of infection with sessile sucking scale on the juniper seed viability after seed phase two and three (SP2 and SP3 respectively) (SE stands for Standard Error).

	SP2				SP3			
	Estimate	SE	z-value	p-value	Estimate	SE	z-value	p-value
Ripening time	-2.7637	0.3934	-7.041	<0.001	0.6628	0.4539	1.46	n.s.
Probability of infection with sessile sucking scale	0.3949	0.2801	1.41	n.s.	0.1777	0.5253	0.338	n.s.

After seed phase two, the viability of seeds that ripen in three or more years reaches the minimum in most populations, whereas seeds that ripen in less than three years still show a strong decrease during seed phase three (Fig. 3-6). However, beside in the populations of Ekulunde, Kleszczele and Markelo, the viability of the seeds with a ripening time of less than three years remained higher than the viability of seeds with a ripening time of three of more years. Nevertheless, a relationship between seed viability after seed phase three and the ripening time was absent (Table 3-3).

3.4 Discussion

The aim of this study was to achieve a better comprehension of the mechanisms behind the low seed viability of common juniper by comparing the seed quality of populations with and populations without recruitment. The most striking result is the progressive and drastic reduction of seed viability over the three seed phases ending with a significantly lower chance for viable seeds with a ripe embryo that are ready for dispersal, in the populations without recruitment. This indicates that an important regenerative bottleneck is possibly situated in seed phase three. The results also suggest that the viability of SP2 seeds is strongly correlated with ripening time. We will firstly discuss the possible reasons for viability loss after each of the three studied seed phases with special attention for the influence of ripening time during seed phase two and three. Next we shortly elaborate on the importance of seed predation.

3.4.1 Seed phase one

During the first seed phase the percentage of viable seeds decreased with up to 50%, mainly because no pollination took place. Amongst conifers, losses of up to 30% due to a lack of pollination are not abnormal (Owens 1995, Owens & Morris 1998). Being a dioecious species, it is possible that the pollination success in common juniper is even lower than in most monoecious trees (Anderson & Owens 2000). This lack of pollination is an important cause of ovule abortion in most coniferous species (Owens 1995). However, in many Cupressaceae, the unpollinated ovules continue to develop normally until the fertilization should take place, leading to a partially formed seed coat and seeds that appear normal from the outside (Owens 1995).

Pollination failure in common juniper can have different causes. Pollen availability can be low due to late frost (Thomas *et al.* 2007), while García *et al.* (2002) found a positive correlation between unpollinated ovules and the amount of precipitation during the pollination period. In addition, a partial pollination drop withdrawal caused by deposition of particles other than juniper pollen (e.g. *Pinus* spp. pollen and inorganic particles) may reduce the probability of successful pollination (Mugnaini *et al.* 2007). Other possible causes of abortion in this phase of development are frost damage, early incompatibility mechanisms and insect damage (Owens 2006). However, the difference between populations with and populations without recruitment was not significant. There are also no indications that common juniper is susceptible for producing inviable pollen (see Box I). Hence, failure during this phase cannot explain the low recruitment.

Box I: Pollen viability

An important phase in the sexual reproductive process of plants is the production of viable pollen. In the male common juniper shrubs, cone initiation starts in autumn (Singh 1978) but only in the next spring, the microsporangia are differentiated (Ottley 1909). After the last division of the archesporial tissue (end of April - beginning of May), the microspore mother cells are formed and each microsporangium consist of a central mass of polygonal microspore mother cells. Very soon the meiotic divisions of the macrospore mother cells begin and after a short time, the tetrads, containing four ripe microspores are formed. When the walls of the spores are formed the mother wall breaks and the spores are set free in the microsporangium. The microspores increase in size and about 15 days after the formation of the microspore mother cells, the pollen are dispersed (Ottley 1909).

Meiosis is an important process during pollen production and normally leads to a huge amount of tetrads. Hence, the presence of a high percentage of aberrant tetrads (e.g. incomplete tetrads, absence of cell walls, incomplete cytokinesis), can indicate that pollen of common juniper is susceptible for disturbances during meiosis.

In this exploratory research, we examined if there were indications that the pollen originating from northern Belgium, a region were common juniper has an extremely low seed viability, shows any abnormalities. We sampled a male cone of a common juniper shrub from a natural population in northern Belgium, crushed it and studied the tetrads under a microscope. A total of 442 tetrads was counted, of which 97.1% were normal. The high percentage of normal tetrads suggests that meiosis is mostly running normal.

A second method to estimate pollen viability is by the size variation of ripe pollen (Kelly *et al.* 2002). The diameter distribution of an amount of pollen should be centralised around one peak. More than one peak indicates a group of pollen with anomalous sizes (e.g. smaller ones for inviable pollen or bigger ones for diploid pollen). Pollination with these pollen could lead to inviable seeds. In this part of the research, we crushed a ripe cone, again from a northern Belgian male shrub, and measured the diameter of the pollen. Fig. 3-7 shows only one peak around an average diameter of 14.15 μm, which is comparable to diameters measured on common juniper pollen by Nepi *et al.* (2005). Hence, most of the pollen had normal sizes and could be considered viable.

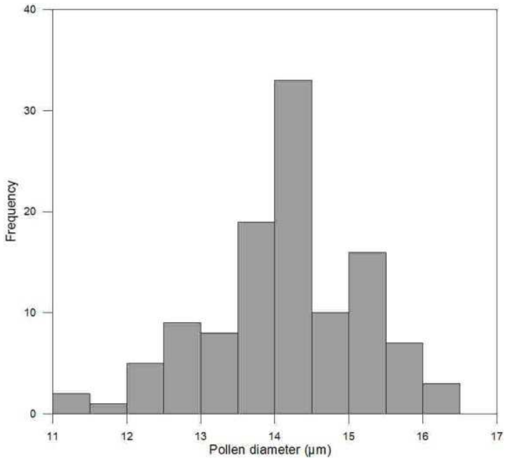


Fig. 3-7. The diameter distribution of the pollen within one male cone.

Although we only studied two male cones, the results of this exploratory research indicate that there are no strong indications that common juniper pollen are susceptible for anomalies during their development. These findings made us decide to not further study this phase in the sexual reproduction of common juniper.

3.4.2 Seed phase two

Nearly equal proportional decreases occur in both groups during the second seed phase. During this phase the megasporogenesis is initiated, the pollen tube reaches the archegonia, fertilization takes place and the first steps in embryo development are taken. There seems to be a complex interaction between the male and female gametophyte before fertilization. Healthy pollen and the pollen tube within the ovule stimulate the ovule development by producing or triggering the production of hormones such as auxins, gibberellins and cytokinins (Fernando *et al.* 2005, Owens *et al.* 2005). Singh (1978) stated that the vicinity of the pollen tube influences the development and maturation of archegonia in several gymnosperms. The growth of the pollen tube possibly requires specific secretions from the female gametophyte (Fernando *et al.* 2005). On the other hand, Owens & Blake (1985) indicated that, in most conifer species, pollen is not required for a normal development of the megagametophyte and archegonia. An extra complication during this phase in common juniper is the variation in ripening time of the seeds. Although the three year cycle is mostly recorded (e.g. Ottley 1909, García *et al.* 2002, Thomas *et al.* 2007), we found that the majority of the seeds (68%) ripened in less than three years. Ward (2010) suggests that the ripening time could be influenced by the growth rate of the pollen tube, which can be influenced by temperature (see also Hedhly *et al.* 2009). In our study, the populations with recruitment experienced harsher winters because of higher latitudes, altitudes or a more continental climate, compared with the populations without recruitment. The two populations with the coldest winters (Ekulunde and Kleszczele) had relatively more seeds that ripened in three years than in two years. This is consistent with the findings of Ward (2010) in the UK, where the ripening period of the seed cones of common juniper was strongly biased to two years in the southern sites, whereas in the northern sites, there was a higher chance of finding cones that needed three years to ripen. Competition for nutrients between the cones can also be an explanation for the longer developing time (Ward 2010) and for seed viability (Fenner & Thompson 2005). Our data show no relation between seed viability and cone density (data not shown) and a negative correlation between the ripening time of the seeds and the viability of SP2 seeds. The latter correlation was also found by Ward (2010). Owens (1991) suggested that the more time the reproductive cycle needs, the more opportunity there is for climatic and other factors to diminish seed viability. For SP2 seeds this could be a plausible explanation. However, no such correlation was found for SP3 seeds (see discussion below).

In *Picea abies*, Owens *et al.* (2001) found a higher rate of abortion of megagametophytes in case of higher temperatures during the period of maturation, fertilization and pro-embryo development. Fertilization itself may be less liable to perturbations. For example, Owens & Morris (1998) found little anomalies during fertilization in *Abies amabilis*. Self- or other incompatibility mechanisms are often put forward as an important reason for seed abortion during this phase (e.g. Kärkkäinen *et al.* 1999, Owens 2006, Owens *et al.* 2008). As a dioecious species, there is no self-incompatibility issue but the pollen in common juniper may still be unsuitable if it is originating from genetically closely related, nearby individuals (Fenner & Thompson 2005). This could be a possible explanation for the decline in the percentage of viable seeds during the second phase, although Vanden Broeck *et al.* (2011) found that genetic diversity within juniper populations in northwestern Europe is still relatively high. In short, the low percentage of viable SP2 seeds can be ascribed to a lack of pollination (which finds its origin during seed phase one) and disturbance of the growth of pollen tube, archegonia and megagametophyte.

3.4.3 Seed phase three

In the third phase, a strong difference in percentage viable seeds was recorded between populations with and without recruitment (13% vs. 3% respectively). Not only did populations without recruitment have a significant lower percentage of viable seeds, the proportional decrease between SP2 and SP3 was also very high. During this phase, the embryo develops a radicle, hypocotyl, plumule and cotyledons (Singh 1978, Bonner 2008). The megagametophyte goes through a phase of meristimatic activity with accumulation of resources such as lipids, starch and protein (Singh 1978, Owens *et al.* 2008); a process that already started prior to fertilization (Bonner 2008). Further maturation of the seed is accomplished with a certain amount of seed drying (Kurz *et al.* 1994). Seeds in which fertilization did not take place can appear viable. For example, for *Abies amabilis* it has been demonstrated that in ovules that were not pollinated, the megagametophyte with fertile eggs develops normally for a certain period after the period in which fertilization should have taken place (Owens & Morris 1998). Hence, abortion in this phase can still be triggered by the degeneration of unfertilized eggs, because the ovule was not pollinated, or by incompatibility mechanisms (Owens 2006, Owens *et al.* 2008).

The correlation found between the viability of SP2 seeds and the ripening time was absent in SP3 seeds. This means that during the third seed phase, the decline in viability was much larger for seeds that ripen in less than three years than for other seeds. This decline is more pronounced in populations without recruitment. Seeds that ripen in three or more years show almost no decline during the third seed phase. Besides the growth of the pollen tube, also the megagametophyte is subject to important changes, e.g. the preparation for food reserve storage (Owens *et al.* 2008) during seed phase two. Hence, it is possible that, because of the accelerated tube growth, the male-female synchrony for successful mating in seeds that ripen in less than three years is disturbed (Herrero 2003), but the expression of this perturbation is delayed until seed phase three. Owens (1995) and Owens & Morris (1998) also state that irregularities causing abortion or abnormal development of a reproductive structure, can take place long before the anomaly has occurred.

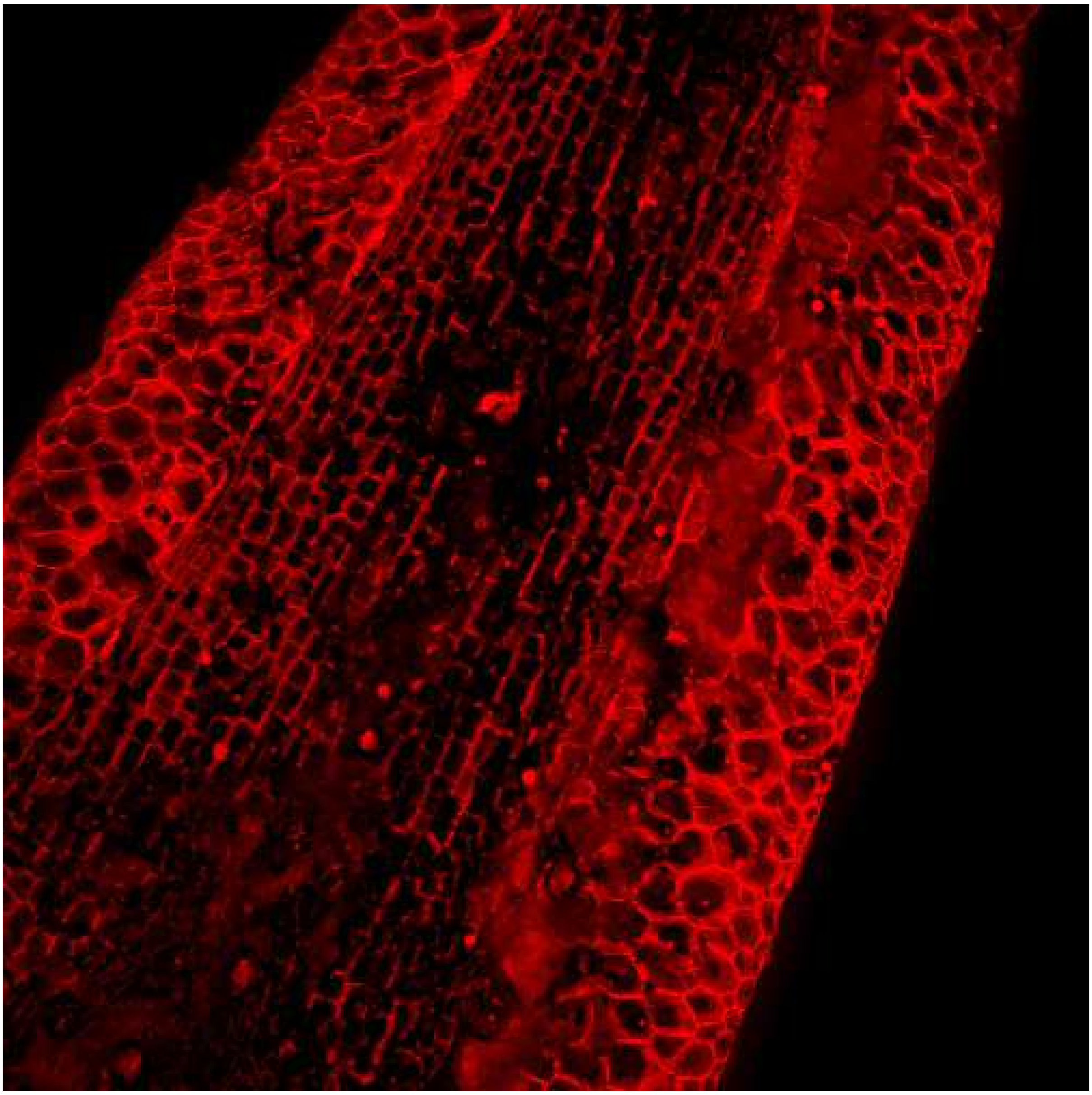
Disturbances during seed phase two can be caused by higher temperatures (Hedhly 2009, Verheyen *et al.* 2009, Ward 2010), shortage of nutrients (Fenner & Thompson 2005) and nitrogen deposition (Verheyen *et al.* 2009), which are all variables that can influence the reproductive biology of common juniper (Ward 2010).

3.4.4 Seed predation

Seed predation is often mentioned as an important cause of viability decrease (e.g. Owens 2006, Verheyen *et al.* 2009). For common juniper, *M. bipunctatus* and mite attacks normally destroy the seeds. However, the low number of attacked seeds and the absence of significant differences in *M. bipunctatus* and mite incidences between populations with and without recruitment indicates that this is likely not responsible for the differences in seed viability between the two population groups. On average, more cones were infested with *Carulaspis* spp. with significant higher attacks on populations without recruitment. García (1998) found that heavy *Carulaspis* spp. attack indicated low seed viability, but here we found no relation between the presence of *Carulaspis* spp. on the cones and the seed viability.

3.4.5 Conclusion

We conclude that anomalies in the ripening of the embryo may be an important factor helping to explain differences in recruitment success of common juniper across Europe. However, looking in more detail at the complex relationship between seed viability and ripening time, we consider it plausible that the causes need to be searched earlier in the seed development. Notably during seed phase two, i.e. between the germination of the pollen and the fertilization. Seeds that ripen in three or more years show already a low viability after seed phase two and seeds that ripen in less than three years show a strong decrease during the third seed phase. This strong decrease could be explained by the accelerated development of the pollen tube and megagametophyte in seeds having a shorter ripening time. Possibly this disturbs the male-female synchrony for successful mating. This may help to explain the negative correlation between temperature and seed viability (Verheyen *et al.* 2009). As temperatures are expected to increase, common juniper may become even more threatened in the coming decades in Europe.



4 Negative correlations between temperature and atmospheric depositions and the seed viability of common juniper (*Juniperus communis*)

After Gruwez R, De Frenne P, De Schrijver A, Leroux O, Vangansbeke P, Verheyen K (2014). Negative effects of temperature and atmospheric depositions on the seed viability of common juniper (*Juniperus communis*). Annals of Botany 113:489-500

Abstract

Environmental change is increasingly impacting ecosystems worldwide. However, our knowledge about the interacting effects of various global-change drivers on sexual reproduction of plants, one of their key mechanisms to cope with change, is limited. Here we study, on populations of poorly regenerating and threatened common juniper (*Juniperus communis*), the correlation of four global-change drivers (rising temperatures, nitrogen deposition, potentially acidifying deposition and altering precipitation patterns) with two key developmental phases during the sexual reproduction, i.e. gametogenesis and fertilization (seed phase two, SP2) and embryo development (seed phase three, SP3), and on the ripening time of the seeds. In 42 populations throughout the distribution range of common juniper in Europe, 11943 seeds of two developmental phases were sampled. Seed viability was determined using seed dissection and related to accumulated temperature (expressed as growing degree days), nitrogen and potentially acidifying deposition (nitrogen plus sulphur) and precipitation data. Precipitation was not correlated the viability of the seeds and on the ripening time. Increasing temperatures were negatively correlated with the viability of SP2 and SP3 seeds and decreased the ripening time. Potentially acidifying depositions were negatively correlated with SP3 seed viability while enhanced nitrogen deposition was linked to lower ripening times. Higher temperatures and atmospheric deposition apparently affected SP3-seeds more than SP-2 seeds. However, this is possibly a delayed effect as juniper seeds develop practically independently, due to the absence of vascular communication with the parent plant shortly after fertilization. We propose that the failure of natural regeneration in many European juniper populations might be attributed to climate warming as well as enhanced atmospheric deposition of nitrogen and sulphur.

4.1 Introduction

In order to get a better understanding in how different global change drivers can influence the sexual reproduction in plants, Hedhly *et al.* (2009) underlined the importance of studying the sensitive phases during reproduction. In this chapter we follow such an approach in the coniferous shrub common juniper (*Juniperus communis* L.) to assess the effects of increasing temperature, altering precipitation and atmospheric depositions across its distribution range.

Verheyen *et al.* (2009) found a negative relationship between seed viability on the one hand and increasing temperature (expressed as mean annual growing degree-days above 0 °C) and enhanced nitrogen deposition on the other hand. In combination with climate warming, changing precipitation patterns are often put forward as important drivers of sexual reproduction in plants (e.g. Owens 1995, Walck *et al.* 2011). Although Verheyen *et al.* (2009) found no relationship between seed viability of ripe common juniper seeds and precipitation, there can still be an influence during other phases of the sexual reproduction.

The most critical phase of predispersal seed development in common juniper occurs during embryo development (seed phase three, cfr. Chapter 3). However, it remains unclear whether the reasons for the failure of embryo development occurs in this phase or in the previous phase of the growth of the pollen tube, gametogenesis, fertilization and early embryo development (seed phase two, cfr. Chapter 3), as different processes during seed development can regulate each other (Fig. 4-1).

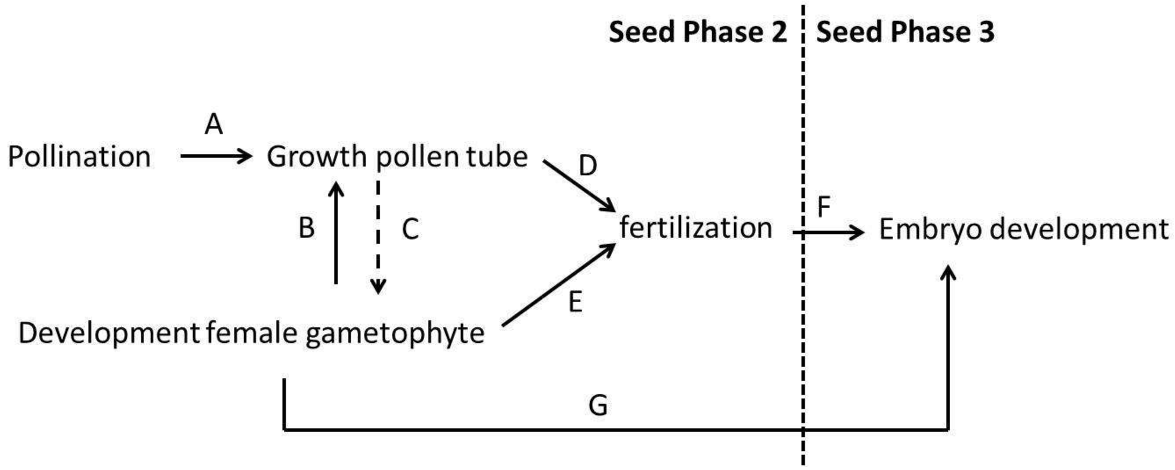


Fig. 4-1. Schematic of the relationships between the key processes during the seed development of *Juniperus communis* (full arrows for important relationships, dotted arrows for less important relationships). Arrows A-G are explained in the text.

Two processes that need to be successful to allow fertilization are pollen tube growth and female gametophyte development (Fig. 4-1 D and E). A prerequisite for pollen tube growth is that pollination supplies healthy pollen (Fig. 4-1 A). There also seems to be an interaction between the pollen tube and the female gametophyte (Fig. 4-1 B and C). In some species, a normal development of the ovule is promoted by the pollen or pollen tube, that triggers the production of hormones such as auxines, gibberellins and cytokinins (Fernando *et al.* 2005; Owens *et al.* 2005). However, many gymnosperms show a normal development of the female gametophyte until shortly after the period of fertilization, even without pollen (Owens & Blake 1986, Owens 1995, Owens & Morris 1998). Growth of the pollen tube on the other hand often requires the presence of a healthy female gametophyte (e.g. Fernando *et al.* 1997, Takaso & Owens 1996, Drews & Koltunow 2011), probably because it provides specific secretions such as electron dense substances from the prothallial cells (Takaso & Owens 1996, Fernando *et al.* 2005) (Fig. 4-1 B). During the pre-fertilization stage, the female gametophyte not only forms the archegonia and egg cells but also prepares, for instance, for seed reserve storage (Owens *et al.* 2008). Thus, the female gametophyte in gymnosperms accumulates nutrients before fertilization (Vuosku *et al.* 2009) and at the moment of fertilization, the megagametophytes have almost reached their full development and there is practically no vascular communication between the seeds and the ovuliferous scales. Therefore, in general, the seeds are autonomous after fertilization (Owens & Blake 1986, Owens 1991) and the megagametophyte nourishes the developing embryo (Durzan & Chalupa 1968, Vuosku *et al.* 2009). Thus, the development of the female gametophyte not only directly influences the fertilization (Fig. 4-1 E) and indirectly the growth of the pollen tube (Fig. 4-1 B), but anomalies during this phase can also lead to nutritional problems during embryo development (Fig. 4-1 G).

In common juniper, an additional complexity is that seeds can ripen in two or in three years. In the latter case, fertilization is postponed for one year. A lag between pollination and fertilization is found in different conifers (e.g. different *Pinus* species; Singh 1978), but the reasons remain unclear. It appears that the pollen tube goes in dormancy while the female gametophyte is slowly developing. Shortly before fertilization, the pollen tube revives due to unknown cues (Williams, 2009). Willson & Burley (1983) suggested that delayed fertilization increases the time for selection of male gametophytes and female archegonia, but they also mentioned the possibility that short reproductive seasons force plants to spread pollination and fertilization over one year (Willson & Burley 1983). However, there is still no consensus. In common juniper, the pattern of seed ripening can be dichotomized, with some seeds required a time interval of a few months and others a full year. These two strategies may appear within the same shrub (Chapter 3). To our knowledge, this dual strategy is absent in other conifers. In addition, a complex relationship between the ripening time of the seeds of common juniper and the seed viability appears to exist. Most seeds ripening in three years already have low viability shortly after fertilization, whereas the viability of seeds ripening in two years decreased mostly during embryo development (Chapter 3).

In the present study, we sampled of the hitherto spatially widest spread of populations (to our knowledge) of common juniper and collected seeds of two different development phases (shortly after fertilization and at the end of embryo ripening). By sampling in 42 populations throughout Europe (from Sweden to Spain and from Ireland to Poland), we are able to take advantage of the wide climatic and deposition gradients (De Frenne *et al.* 2013) in this area. We examined the influence of four global-change drivers (increasing temperature, nitrogen deposition, potentially acidifying depositions and altering precipitation patterns) on the seed viability of common juniper after both seed development phases and on the ripening time. The aim of the present study is therefore to test the following hypotheses: (1) that the influence of global-change drivers on seed viability is more pronounced after seed phase two, which indicates that this phase is more vulnerable and (2) that these global-change drivers are determinants of which ripening time strategy occurs in seed.

4.2 Methods

4.2.1 Population, shrub and seed characteristics and sampling

Seeds of 42 populations across the species' distribution range in Europe (Fig. 4-2 A and Appendix B1 and B2) were sampled in autumn of 2008 and 2010 (8 and 34 populations respectively). Populations consisted of at least 30 individual shrubs growing in unshaded conditions (i.e., not below other tree species). In each population three to eight (with a median of five) cone bearing shrubs were randomly selected. Per shrub, three branches were randomly selected, of which on average 28.7 (± 9.4 SD) SP2 seeds and 23.2 (± 9.9 SD) SP3 seeds were sampled.

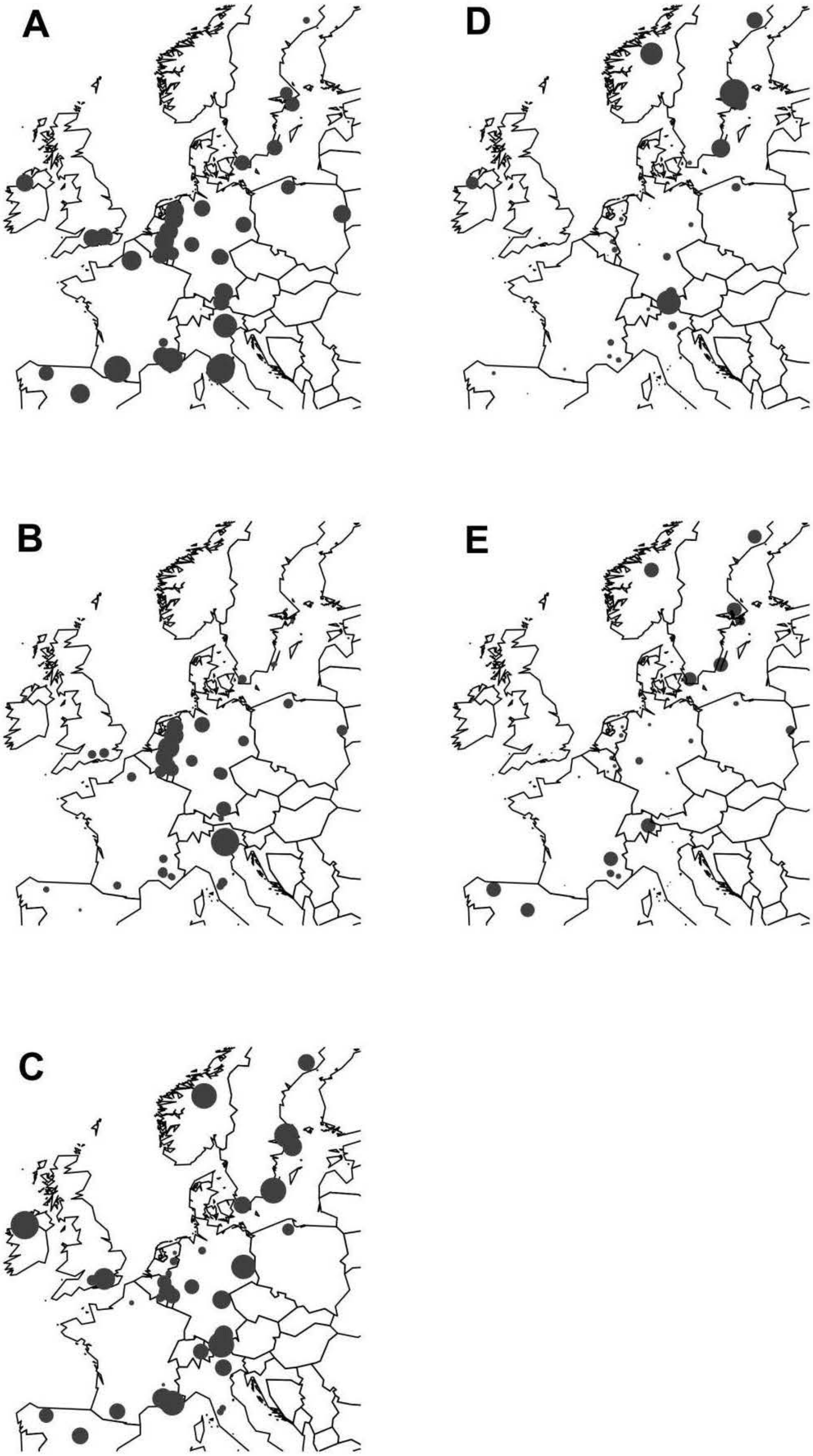


Fig. 4-2. The locations and characteristics of the 42 sampled European common juniper (*Juniperus communis*) populations. Per map, the size of the dots is a relative measure for the GDD>0°C (growing degree days above 0 °C base temperature) during embryo development (A); the amount of potentially acidifying deposition (N + S; keq ha^{-1} year^{-1}) (B); the percentage viable SP2 (C) and SP3 seeds (D) per population; and the percentage seeds that ripen in three instead of two years (E).

Different characteristics were measured and estimated on three different levels: population, shrub and seed. The age of the seeds was deduced from the age of the woody branches on which the cones (containing the seeds) were growing (the cones are always one year younger than the wood). The age of the wood can be determined by counting the growing shoot internodes that are separated by the annual bud scars. By taking the seed phase and the age of the seed into account, we then calculated the ripening time of the seeds (see Chapter 3). For every seed, the number of growing degree days above 0 °C base temperature ($GDD_{>0°C}$; cf. Hall *et al.* 2002) was calculated for three important processes during seed development: pollination, fertilization and embryo development. Depending on the collection date, seed phase and the ripening time, data of different years and seasons were used (Appendix B3). Daily minimum and maximum temperatures of each population were obtained from the nearest weather stations (see Appendix B1) and used to calculate the $GDD_{>0°C}$. When the population and the weather station had different altitudes, a mean adiabatic lapse rate of 5.5 $K \cdot km^{-1}$ (Körner 2007) was applied. The $GDD_{>0°C}$ ranged between 257.6 and 1633.8 with an average of 979.5 (± 245.6 SD) for the spring of pollination, between 775.7 and 2164.1 with an average of 1554.8 (± 243.8 SD) for the summer of fertilization and between 1217.9 and 5074.7 with an average of 3344.8 (± 786.7 SD) for the year of embryo development (Fig. 4-2 A). As temperatures during winter were highly correlated with temperatures for spring of pollination, summer of fertilization and year of embryo development (each time a $p < 0.001$ and a Pearson’s correlation index of 0.73, 0.49 and 0.79 respectively), we decided to use temperature data from periods during which the seed development was active. To illustrate the large-scale temperature gradients in the study region, we refer to Appendix B4.

Nitrogen and sulphur deposition data were obtained from the European Monitoring and Evaluation Programme database (http://www.emep.int). EMEP is the ‘Co-operative Programme for Monitoring and Evaluation of the Long-range Transmission of Air pollutants in Europe’ and provides scientific information on the emission, transport and deposition of air pollutants. Here, data for 2008 were used: total (wet + dry) inorganic nitrogen ($NH_x + NO_y$) depositions expressed as $kg.ha^{-1}.year^{-1}$ and potentially acidifying ($NH_x + NO_y + SO_x$) depositions expressed as $keq.ha^{-1}.year^{-1}$ in 50 x 50 km grid cells covering Europe. Nitrogen depositions ranged from 1.84 to 36.05 $kg.ha^{-1}.year^{-1}$ with an average of 12.21 $kg.ha^{-1}.year^{-1}$ (± 6.9 SD). Potentially acidifying depositions ranged from 0.20 to 3.03 $keq.ha^{-1}.year^{-1}$ with an average of 1.17 $keq.ha^{-1}.year^{-1}$ (± 0.60 SD) (Fig. 4-2B).

Next, the annual amount of precipitation in the year preceding the time of sampling was calculated per population using the monthly precipitation data from Climatic Research Unit (CRU) time-series datasets (Harris *et al.* 2013). Yearly precipitation ranged from 467.9 to 2280.8 mm.year^{-1} with an average of 858.9 mm.year^{-1} (± 301.1 SD).

Correlation between temperature variables, atmospheric depositions and annual precipitation were low, while the correlation between nitrogen deposition and potentially acidifying deposition was very high (Appendix B5).

Finally, for each population we estimated two soil characteristics in different classes: texture of the first 50 cm (sandy, sandy loam, loamy, clayey) and bedrock type (calcareous vs. non-calcareous).

At the shrub level, three characteristics: proportion of needle loss, cone density and plant height, were estimated. Different classes of needle loss (<20%, <40%, ≥40%) were used as an indicator for the health of each shrub. Cone density was subdivided in three classes: low (the cones appear scattered and it is difficult to find any), normal (the cones appear scattered, but they are rather abundant), dense (large clusters of cones are abundant). Finally, the height of each shrub was measured and classified in five different height classes (<0.5 m, <1 m, <2 m, <3 m and ≥ 3m).

4.2.2 Seed analyses

The viability of all sampled seeds was assessed by means of stereoscopic observations of dissected seeds (6609 seeds for SP2 and 5333 seeds for SP3). Seed analysis in this research was similar to the seed analysis in Chapter 3. Hence, seeds that had no visible signs of anomalies were considered to have the potential to develop to the next phase and are further referred to as ‘viable seeds’. Viable SP2 seeds presented a megagametophyte and nucellus consisting of green-white and moist tissue, not completely filling the space within the seed coat (Chapter 3). Viable SP3 seeds consisted of an embryo and megagametophyte with a smooth, white and moist surface. In this phase, almost all space within the seed coat is filled (Chapter 3). In both SP2 and SP3, seeds were occasionally damaged by mites (e.g. *Trisetacus quadrisetus* [Acarina, Eriophyiidae]) or by the seed predator chalcid *Megastigmus bipunctatus* (Hymenoptera, Torymidae; see Roques & Skrzypczynska (2003) for a review of the seed-infesting chalcids of the genus *Juniperus*). The content of seeds attacked by mites is completely distorted and, mostly, the mites are still present. Damage by *M. bipunctatus* could be recognised by the granular content, an exit hole or the presence of larvae. For both seed phases, seeds were scored on the base of viability (non viable or viable), presence of mites and *M. bipunctatus* (absent or present) and the ripening time (two or three years). Mean infection rates with mites and *M. bipunctatus* were calculated for each shrub and population.

4.2.3 Data analysis

To study the relationships between the seed viability of SP2 seeds (viable or not), SP3 seeds (viable or not) and the ripening time (2 or 3 years; seed-level data throughout) on the one hand, and the climatic, environmental, soil, shrub and seed (ripening time) variables (fixed-effect terms) on the other hand, generalised linear mixed modelling with binomial distributions was applied, using the *glmmML* function of the *glmmML* library and the *lmer* function of the *lme4* library in R 2.15.1 (R Development Core Team 2012). Ripening time was only included in the models concerning the viability of SP2 and SP3 seeds. Populations were treated as clusters within the *glmmML* function and as random effects within the *lmer* function. In a first step, all variables were entered in the model on a one-by-one basis. Per dependent variable (viability of SP2 and SP3 seeds and ripening time), variables with a significance level of 0.1 were selected for multivariate modelling. Autocorrelation between the selected variables was checked by calculating the variance inflation factor (Quinn & Keough 2002). In case of autocorrelation (notably between $GDD_{>0°C}$ during pollination, $GDD_{>0°C}$ during fertilization and $GDD_{>0°C}$ during embryo development and between nitrogen deposition and potentially acidifying depositions), only the most significant variables were selected.

Subsequently, all possible models for the three dependent variables (i.e. built by each combination of the selected fixed-effects terms, giving 384 models in total) were compared using the Akaike's Information Criterion, adjusted for sample size (AIC_c) (Hurvich & Tsai 1989). The ΔAIC_c of a model was then calculated as the difference between the AIC_c of the model with the best fit and the AIC_c of that model. Models with $\Delta AIC_c \leq 4$ were considered equivalent (Bolker 2008). To determine the relative importance of the explanatory variables, the sum of Akaike weights of the set of all top models (Delta $AIC_c \leq 4$) in which the variable appeared (Burnham & Anderson 2002) was used. The Akaike weight reflects the weight of evidence in support of a particular model relative to the entire model set, and varies from 0 (no support) to 1 (complete support). For each explanatory variable the relative importance was calculated by summing the Akaike weights of the models containing the variable. Finally, the averaged parameters of the top models were calculated using the model averaging function based on the AIC_c of the MuMin package in R). In addition, for each dependent variable, we calculated the r^2 for the model with the lowest AIC_c using the following function: *r2.corr.mer<-function(m){lmfit<-lm(model.response(model.frame(m)) ~ fitted(m)); summary(lmfit)$r.squared}*.

Finally, to visualize the effects of temperature, nitrogen deposition and potentially acidifying depositions on seed viability and ripening time, we calculated the proportion of viable SP2 and S3 seeds and the proportion of seeds that ripened in three years per population. A similar procedure was followed for shrub height, cone density and needle loss, where the proportion per class was calculated.

4.3 Results

As expected, seed viability declined between seed phase two and three (Fig. 4-2). The average percentage of viable SP2 seeds per population was 38.2% (+/-18.6 SD) with a minimum of 2.3% and a maximum of 73.8%. For SP3 seeds the average was 10.9% (+/-14.6% SD) with a minimum of 0% and a maximum of 58.2%. The seed viability exhibits a large variability for both SP2 seeds and SP3 seeds. For example, 12 populations (in Belgium, France, Germany, Italy, the Netherlands, Spain and the UK) had extremely low percentages (<1%) of viable SP3 seeds. Conversely, populations with higher percentages of viable SP2 seeds (>40%) were situated in Scandinavia, on the axis of eastern Germany towards northeastern Italy, on the axis north-central Spain towards south-eastern France, in Ireland and the southwest of the UK (Fig. 4-2 C). After SP3 most of the Scandinavian populations still had relatively high percentages of viable seeds (>20%), together with three populations in southern and central Germany and Austria and the Irish population (Fig. 4-2 D).

Infection rates with mites and *M. bipunctatus* were relatively low for populations (mean 8.3% and 3.1%, respectively), although infection rates of individual shrubs can be significant (e.g. 93.3% and 76.9%, respectively). In only one population, more than half of the seeds were infected with mites (53.4%), while *M. bipunctatus* infection rates always stayed below 20%.

Most of the seeds ripened in two years (64.5%). Fourteen populations had more than 90% of their seeds ripening in two years and for nine populations this percentage dropped below

10%. Seeds with a three year ripening time were mainly found in Scandinavia, but populations with high percentages were also present in Spain and southeastern France (Fig. 4-2 E).

Selection of the variables for multivariate modelling lead to different results for the viability of SP2- and SP3-seeds and ripening time. For the viability of SP2-seeds, the factors precipitation, mite infection rate, type of bedrock, soil texture, needle loss and ripening time did not have a significance level lower than 0.1. These variables were therefore considered as not important and excluded from the model selection procedure. In a similar way, precipitation, mite and *M. bipunctatus* infection rate, type of bedrock, soil texture and needle loss were excluded for SP3-seeds and precipitation, mite infection rate and soil texture for ripening time. In addition, autocorrelation occurred between the temperature variables, and between the atmospheric deposition variables, resulting in a selection (on the base of the factor with the highest significance level) of $GDD_{>0°C}$ during pollination and potentially acidifying deposition for SP2-seeds; of $GDD_{>0°C}$ during embryo development and potentially acidifying deposition for SP3-seeds; and of $GDD_{>0°C}$ in the year before sampling and nitrogen deposition for ripening time.

The most important variables affecting the viability of SP2 seeds were temperature ($GDD_{>0°C}$ during pollination), infection rate with *M. bipunctatus* on a population level and shrub height, which all had a negative influence (Table 4-1, Fig. 4-3 A & Fig. 4-4 A). Potentially acidifying deposition had only a marginally negative influence (Table 4-1, Fig. 4-3 B). The ripening time strategy for a given seed had no influence on the viability of SP2-seeds. However, since the r^2 was relatively low (0.16), a large part of the variation in the data is not explained by our predictor variables.

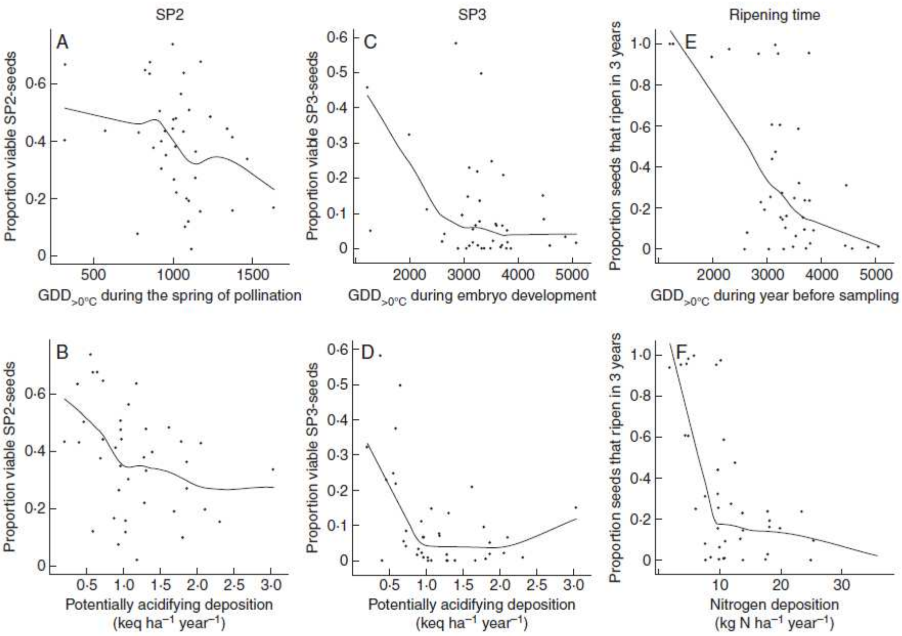


Fig. 4-3. Relationships between accumulated temperature (GDD>0°C during the spring of pollination for seed phase two, GDD>0°C during embryo development for seed phase three and GDD>0°C during the year before sampling for the ripening time) (A, C, E), potentially acidifying deposition (keq ha-1 year-1) for seed phase 2 and 3, and nitrogen deposition (kg N ha-1 year-1) for ripening time (B, D, F) on the one hand, and viability of SP2-seeds (left column), viability of SP3-seeds (middle column) and the seed ripening time (right column). A smoothing spline is fitted to the continuous data.

The patterns were slightly different for SP3 seeds (Table 4-2). Here both potentially acidifying depositions and temperature (GDD$_{>0°C}$ during embryo development) were negatively correlated with seed viability (Fig. 4-3 C & D). In addition, also their interaction was important and indicated that the negative correlation with temperature was more pronounced in populations with a lower potentially acidifying deposition. In addition, shrub height and cone density showed an important negative and positive correlation, respectively (Fig. 4-4 D & E). Seeds that ripened in three years had a slightly greater chance of being viable than seeds that ripened in two years, but this effect was less pronounced (Table 4-2). For SP3, the model with the lowest AIC$_c$ had an r² of 0.24.

Table 4-1. Model selection statistics for the analysis of the effects of GDD>0°C during the spring of pollination, potentially acidifying deposition, their interaction, infection rate with M. bipunctatus, cone density and shrub height on the viability of seed phase 2 seeds. Df: degrees of freedom; ΔAICc: difference in values of the corrected Akaike Information Criterion between a model and the best model; Weight: Akaike weight indicating the relative support for the model; Importance: the relative importance of the explanatory variables based on the sum of the Akaike weights of the models in which the variables appear.

Intercept	Growing degree days (GDD) pollination	Potentially acidifying deposition (PAD)	GDD:PAD	Infection rate with *M. bipunctatus*	Cone density	Shrub height	Df	ΔAICc	Weight
3.50	-3.09E-03			-1.19E+01		+	8	0	0.48
3.55	-3.02E-03	-9.79E-02		-1.21E+01		+	9	1.85	0.19
3.40	-3.12E-03			-1.17E+01	+	+	10	1.92	0.18
3.96	-3.41E-03	-4.47E-01	3.27E-04	-1.24E+01		+	10	3.72	0.08
3.45	-3.06E-03	-9.51E-02		-1.19E+01	+	+	11	3.78	0.07
Importance →	1.00	0.34	0.07	1.00	0.26	1.00	-	-	-

+: factorial variable is included in the model

Table 4-2. Model selection statistics for the analysis of the effects of GDD>0°C during embryo development, potentially acidifying deposition, their interaction, cone density and shrub height and ripening time on the viability of seed phase 3 seeds. Df: degrees of freedom; ΔAICc: difference in values of the corrected Akaike Information Criterion between a model and the best model; Weight: Akaike weight indicating the relative support for the model; Importance: the relative importance of the explanatory variables based on the sum of the Akaike weights of the models in which the variables appear.

Intercept	Growing degree days (GDD) embryo development	Potentially acidifying deposition (PAD)	GDD:PAD	Cone density	Shrub height	Ripening time	Df	ΔAICc	Weight
4.70	-2.07E-03	-6.95E+00	1.72E-03	+	+	+	12	0	0.65
5.15	-2.16E-03	-7.18E+00	1.77E-03	+	+		11	1.20	0.36
Importance →	1	1	1	1	1	0.65	-	-	-

+: factorial variable is included in the model

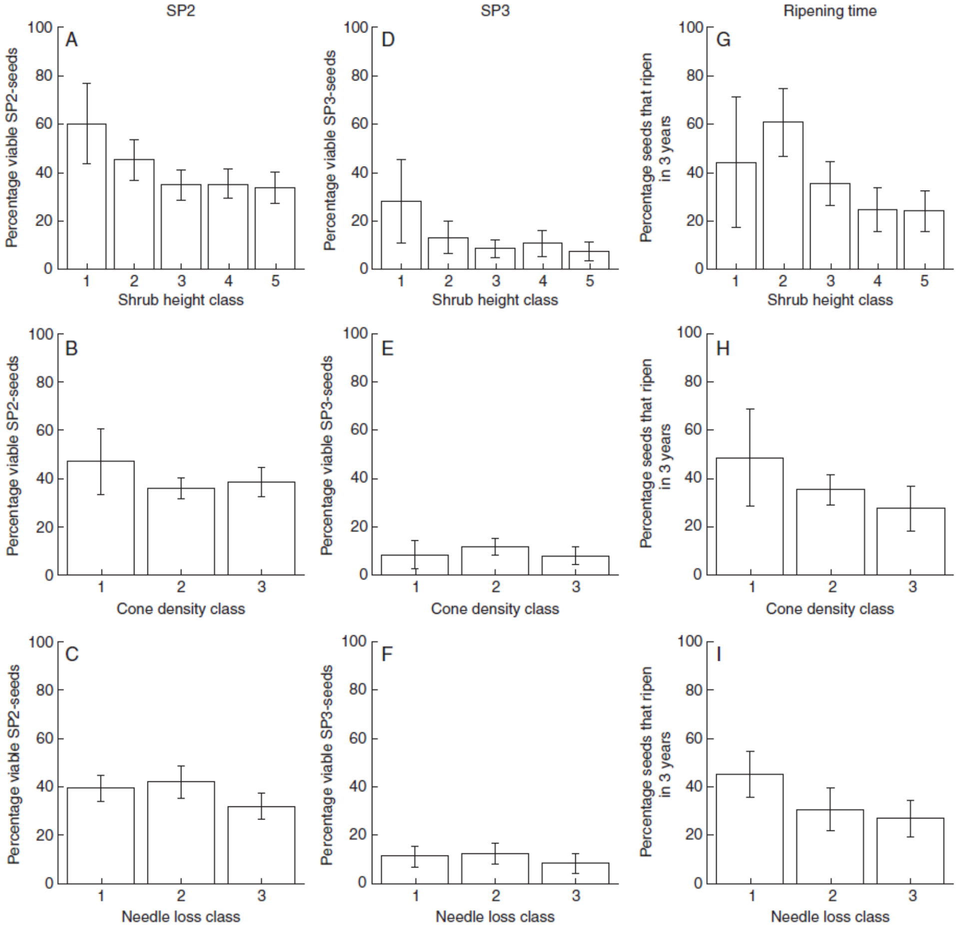


Fig. 4-4. Relationships between shrub height (A, D, G), cone density (B, E, H) and needle loss (C, F, I) on the one hand, and viability of SP2-seeds (left column), viability of SP3-seeds (middle column) and the seed ripening time (right column). Shrub height classes: 1: <0.5 m, 2: , <1 m, 3: <2 m, 4: <3 m, 5: ≥ 3m; needle loss classes: : 1: <20%, 2: <40%, 3: ≥ 40%; cone density classes: : 1: low, 2: normal, 3: dense.

Ripening time was mostly determined by nitrogen deposition, the bedrock type, cone density, shrub height and needle loss (Table 4-3, Fig. 4-3 F and Fig. 4-4 G-I). Seeds originating from populations with a higher nitrogen deposition or growing on soil with a calcareous bedrock more often had a ripening time of two years. Shrubs with a greater cone density had more seeds that ripened in two years and vice versa for taller shrubs or those with a larger needle loss. Higher temperatures (GDD$_{>0°C}$ during embryo development) led to shorter ripening times, but this effect was less pronounced (Fig. 4-3 E). The interaction between temperature and nitrogen deposition was of minor importance. The model with the lowest AIC$_c$ explained almost 50 % of the variation ($r^2 = 0.49$).

Table 4-3. Model selection statistics for the analysis of the effects of GDD>0°C during the year before sampling, nitrogen deposition, their interaction the, bedrock type, cone density, shrub height and needle loss on the ripening time of the seeds. Df: degrees of freedom; ΔAICc: difference in values of the corrected Akaike Information Criterion between a model and the best model; Weight: Akaike weight indicating the relative support for the model; Importance: the relative importance of the explanatory variables based on the sum of the Akaike weights of the models in which the variables appear.

Intercept	Growing degree days (GDD) year before sampling	Nitrogen deposition	GDD:ND	Bedrock type	Infection rate with *M. bipunctatus*	Cone density	Shrub height	Needle-loss	Df	ΔAICc	Weight
6.18	-2.29E-03	-5.53E-01	1.06E-04	+	1.58E+01	+	+	+	15	0	0.22
2.14	-1.20E-03	-1.56E-01		+	1.75E+01	+	+	+	14	0.11	0.21
3.17	-1.47E-03	-1.32E-01			2.30E+01	+	+	+	13	1.08	0.13
8.24	-2.69E-03	-7.14E-01	1.43E-04	+		+	+	+	14	1.15	0.13
7.27	-2.56E-03	-5.18E-01	1.03E-04		2.00E+01	+	+	+	14	1.22	0.12
-1.91		-1.88E-01		+	1.96E+01	+	+	+	13	1.64	0.10
2.84	-1.21E-03	-1.84E-01		+		+	+	+	13	2.67	0.06
-1.15		-2.18E-01		+		+	+	+	12	3.99	0.03
Importance →	0.87	1	0.47	1	0.78	1	1	1	-	-	-

+: factorial variable is included in the model

4.4 Discussion

The aim of this study was to achieve a better understanding of the influence of four global-change drivers (increasing temperatures, enhanced nitrogen and potentially acidification deposition and altering precipitation patterns) on the viability and ripening time of common juniper seeds (*Juniperus communis*). We focussed on two key seed developmental phases, i.e., growth of the pollen tube, megagametogenesis, fertilization and early embryo development (seed phase 2, SP2) and ripening of the embryo (seed phase 3, SP3), that were identified as crucial for the sexual reproductive cycle of this species (Chapter 3). Both needle loss and shrub height (as a rough proxy for age, Breek 1978, Forbes & Proctor 1986) can be seen as a measure for senescence. Cone density on the other hand relates to the vitality of the shrub. This can explain the relationships between cone density, shrub height and seed viability. Precipitation was shown to have no influence. In the following sections, we focus on the important effects of temperature and enhanced atmospheric depositions on seed viability of common juniper.

4.4.1 Temperature effects

We showed that increasing temperatures were negatively correlated with seed viability of both SP2 and SP3 seeds of common juniper and promote a ripening time of two years. Different patterns can be seen between the two studied developmental phases. For the viability of the SP2 seeds, the $GDD_{>0°C}$ during springtime were most important, while, for the SP3 seeds, only the $GDD_{>0°C}$ during embryo development showed a significantly negative correlation.

Our results differ from what is found in other studies on conifers where a positive relationship between higher temperatures and seed viability was revealed (e.g. Despland & Houle 1997, Noland *et al.* 2006, Meunier *et al.* 2007). However, the latter studies were often performed at the northern distribution range limits, whereas our study included common juniper populations from a wider geographical area. Low temperatures and late frosts during pollination are often mentioned as a reason for failing pollination and ovule abortion during further seed development (e.g. Owens 1995, Thomas *et al.* 2007). Therefore, a lack of pollination cannot explain the negative relationship between seed viability and the temperature during the spring.

During SP2, there are two processes (the development of the female gametophyte and growth of the pollen tube) that can help to explain the negative effects of temperature on seed viability (Fig. 4-1).

First, higher temperatures can induce abnormalities in the female gametophyte (Franz & Jolliff 1989, Kozai *et al.* 2004) or other female structures (Saini *et al.* 1983, Hedhly *et al.* 2003, Hedhly *et al.* 2004, Hedhly *et al.* 2005), which can lead to abortion of the seed. Second, little is known about the direct effects of higher temperatures on the viability of the germinating pollen (but see Young *et al.* 2004, Steinacher & Wagner 2012). However, several studies hypothesised that the ovule and female gametophyte might be important regulators of pollen tube growth (e.g. Gifford & Foster 1989, Takaso & Owens 1996, Fernando *et al.* 2005, Drews & Koltunow 2011). Thus, through influencing the female gametophyte, increased temperatures can have an indirect effect. In addition, due to their separate influences on the pollen tube and female gametophyte, high temperatures also have detrimental effects on the male-female synchrony in the pre-ferilization phases (Zinn *et al.* 2010, Hedhly 2011). Hence, both mechanisms, i.e. the negative influence of higher temperatures on the viability of the female gametophyte and the different effects on the growth speed of both female gametophyte and pollen tube, may lead to unviable SP2 seeds.

Higher temperatures during the spring of the pollination and the summer of the fertilization seem to have no influence on viability of SP3-seeds. Perhaps cones with aborted seeds have been shed by the third year, which can mask these effects. On the other hand, $GDD_{>0°C}$ during embryo development had a negative influence on seed viability. Possibly, higher temperatures may disrupt the meristimatic activity of the female gametophyte with accumulation of resources such as lipids, starch and proteins (Singh 1978, Owens *et al.* 2008) and the nutrition and growth of the embryo. Owens *et al.* (2001) and Cross *et al.* (2003), for instance, found a higher rate of abortion in *Picea abies* and *Linum usitatissimum* under higher temperatures during embryo development. An indirect negative effect of temperature on the viability of SP3 seeds due to malfunctions during the pre-fertilization development of the female gametophyte could not be detected as $GDD_{>0°C}$ during that period had no significant effects.

Warmer temperatures during the last year before sampling (for SP2 and SP3 seeds) led to shorter ripening times. The negative correlation of temperature with ripening time can be explained by an altered development of the male and female gametophytes and their interactions. As common juniper can be considered a cold adapted species (*cf.* its relatively northerly distribution range and the assumption of its survival in Central Europe during the last glacial maximum; Michalczyk *et al.* 2010), there is a probability that the mechanism of delayed fertilization to overcome shorter reproduction seasons has developed in this species (Willson & Burley 1983). Longer reproduction seasons due to higher temperatures can reduce the need for such mechanisms, leading to a higher chance of shorter ripening times.

To further our understanding of the role of higher temperature in the described processes, research on a biochemical level is needed.

4.4.2 Effects of nitrogen and potentially acidifying deposition

Acidifying depositions appear to be negatively correlated to seed viability after seed phase three. We can assume that nitrogen deposition has a similar effect as both variables were highly correlated. This correlation was almost absent after seed phase two. In addition, the correlations of temperature and potentially acidifying depositions had a significant interaction after seed phase three: in populations with low potentially acidifying depositions, the negative correlation of temperature was pronounced, while the correlation was slightly reversed in populations with high depositions. Seeds sampled from individuals growing in areas with high nitrogen depositions also ripened more in two instead of three years.

Our results correspond with different studies demonstrating that nitrogen (e.g. Vergeer *et al.* 2003, Li *et al.* 2011) or potentially acidifying depositions (Wertheim & Craker 1987, Feret *et al.* 1990, Munzuroglu *et al.* 2003, Vergeer *et al.* 2003) negatively affect plant performance in terms of seed quality. However, other studies have shown that nitrogen depositions can also increase seed quality (e.g. Drenovsky & Richards, 2005).

Nitrogen and potentially acidifying depositions can influence seed viability in a direct way by creating nutrient imbalances and causing decreased uptake and leaching of cations including K^+, Ca^{2+} and Mg^{2+} in the plant (Bobbink *et al.* 1992, Krupa 2003), leading to nutritional deficiencies (e.g. Pearson & Stewart 1993). As for the influence of temperature, the biochemical mechanisms behind the effects on seed viability need more research. For example, Ca^{2+} plays a role in the control of conifer pollen tube growth (Fernando *et al.* 2005) and detoxification products such as arginine, needed in case of a higher uptake of NH_4^+ and NH_3 through canopy exchange (Krupa 2003), can have disturbing effects (Durzan & Chalupa 1968, Durzan 2002).

Nitrogen and potentially acidifying depositions can also indirectly influence plant performance as it causes a reduction of mycorrhizae associations in several species (Malcová *et al.* 1999, Krupa 2003). For common juniper, symbiosis with mycorrhiza (especially with arbuscular myccorhiza (AM), Thomas *et al.* 2007) can be important; Bakker (1988) suggested a possible relationship between presence of mycorrhiza and the viability of *J. communis* shrubs.

Our results show a correlation with potentially acidifying depositions only after seed phase three. One would expect a better correlation after seed phase two since, as in most conifers, seeds are largely autonomous shortly after fertilisation. However, it is possible that anomalies during seed phase two (e.g. badly developed megagametophyte due to nutrient deficits) only lead to seed abortion during seed phase three, when the megagametophyte nourishes the developing embryo. Similarly, nutrient imbalances and signalling disturbance can also alter the ripening time of the seed development.

There was no correlation between ripening time and seed viability after seed phase two and of it was of much less importance than the correlation between potentially acidifying depositions and temperature after seed phase three. Thus, there is no clear evidence that the potentially negative effects of increased temperatures, nitrogen depositions and potentially acidifying depositions on seed viability are acting through changed ripening duration of the seeds. For both SP2 and SP3-seeds, only 16 to 24 % of the variation was explained by the models. Hence, there are still other causes possible for low seed viability (e.g. microclimate).

4.4.3 Conclusions

The negative correlations between temperature, nitrogen deposition and potentially acidifying deposition and the viability of both SP2 and SP3-seeds of common juniper suggest that these global-change drivers potentially affect different key processes of the sexual reproductive cycle of *Juniperus communis* including pollen tube growth, megagametogenesis and embryo development. Thus, our findings put forward that the failure of natural regeneration in many European juniper populations might be attributed to climate warming as well as high atmospheric deposition of nitrogen and sulphur.

5 Do climate warming and atmospheric deposition correlate negatively with seed viability of common juniper (*Juniperus communis*) via their impact on the nutrient status of the plant?

After Gruwez R, De Frenne P, De Schrijver A, Vangansbeke P, Verheyen K. Climate warming and atmospheric deposition affect seed viability of common juniper (Juniperus communis) via their impact on the nutrient status of the plant. Ecological Research, accepted with major revisions

Abstract

Global environmental change is increasingly affecting species worldwide. One of the emblematic victims among plants is common juniper (*Juniperus communis*). Many populations of common juniper throughout its distribution range are declining. The relative lack of germinable seed production, resulting in low probabilities for successful natural regeneration, is one of the main reasons for this decline. Climate warming and elevated atmospheric depositions are negatively correlated with seed viability of common juniper, but the driving mechanisms remain unclear. One of the possible pathways is via the influence of these global-change drivers on the overall nutrient status of the plants, which, in turn, can affect seed viability. Here we report the results of a sampling campaign of needles and seeds in 20 juniper populations spread across Europe. First, we studied the correlation between increasing temperature, elevated potentially acidifying atmospheric depositions and altering precipitation on the one hand and the needle nitrogen (N), phosphorous (P), carbon (C), sulphur, potassium, calcium (Ca) and magnesium (Mg) concentrations and C:N and N:P ratios on the other hand. Second, we assessed the relationship between needle composition and seed viability. Both temperature and potentially acidifying depositions were correlated with needle nutrient concentrations. Changing nutrient availability, leaching and the dilution effect probably altered the nutrient status of the shrubs. Low needle P, Ca and Mg concentrations were related to low seed viability. Thus, a shortage of these key elements during seed development and seed nutrient storage, can lead to anomalies and seed abortion, thereby helping to explain the low seed viability of juniper across Europe.

5.1 Introduction

In Chapter 4 we found that mainly two global-change drivers, i.e. climate warming and enhanced airborne depositions of potentially acidifying substances such as nitrogen (N) and sulphur (S), were negatively correlated with seed viability of common juniper. Moreover, the negative correlations with increasing temperatures were found both after fertilization and during embryo development. Correlations with potentially acidifying depositions were, however, only pronounced after embryo development. The mechanisms behind these correlations, however, remain unclear. Although we found no correlations between precipitation and seed viability (see Chapter 4), studying its influence can still be important as precipitation correlated events such as drought can affect the sexual reproduction in plants (Demirtas *et al.* 2010).

Several studies showed mostly negative correlations between foliar concentrations of N and phosphorous (P) and temperature (Reich & Oleksyn 2004, Han *et al.* 2005, Zheng & Shangguan 2007, Kang *et al.* 2011; but see Yuan & Chen 2009,) and positive correlations with N deposition (Innes 1995, Thimonier *et al.* 2010, Sardans *et al.* 2011, Blanes *et al.* 2013). Far less is known about the effects of these global-change drivers on tissue nutrient concentrations of potassium (K), magnesium (Mg), S and calcium (Ca) (but see Sardans *et al.* 2011).

Macronutrient concentrations in foliage, in turn, can influence seed production and quality. For example, foliage N, P and K concentrations showed positive correlations to the number of flowers in *Malus* spp. (Marschner 1995) and to the number of conelets and the seed weight in *Pinus sylvestris* (Karlsson & Örlantder 2002). Indeed, the macronutrients N, P, S, K, Ca and Mg play an important role in plant growth and plant functioning (Marschner 1995). It is well known that N and S compounds accumulate as reserves during seed development (mostly as proteins), but also P, Mg, K and Ca are sequestered within mature seeds (Lott *et al.* 1995). Beside as a reserve, K, Ca and Mg also fulfil a regulative role (e.g. osmoregulation, cell extension and cell wall stabilisation) (Marschner 1995). For common juniper, Lucassen *et al.* (2011) found a relationship between the chemical composition of the needles and the seeds on the one hand, and the abundance of seedlings in Dutch populations (i.e. positive for K and P concentrations and negative for aluminium concentrations). Hence, a possible pathway to explain why temperature and atmospheric depositions are negatively correlated with on seed viability is via their potential influence on macronutrient concentrations in the plant (which are represented by the concentrations in the needles.

Here we report the results of a large-scale sampling campaign of needles and seeds of two development phases (that is, seeds sampled shortly after fertilization and at the end of embryo ripening) in 20 common juniper populations throughout Europe (from Sweden to Spain and from the United Kingdom to Poland). We took advantage of the wide climatic and atmospheric deposition gradients (De Frenne *et al.* 2013) in this area to study how three global-change drivers (increasing temperature, potentially acidifying depositions and altering precipitation) were correlated with the macronutrient concentration in the needles. In addition, we link the macronutrient concentration in the needles to the viability of the seeds after both seed phases. We specifically assessed the following hypotheses: (1) the three global-change drivers are correlated with the macronutrient concentrations in the common juniper needles and (2) these macronutrient concentrations are, in turn, related to seed viability of juniper.

5.2 Methods

5.2.1 Sampling

Seeds of 20 populations across the species' distribution range in Europe (Fig. 5-1 and Appendix C1 and C2) were sampled in autumn of 2010. Populations consisted of at least 30 individual shrubs growing in unshaded conditions (i.e., not below the canopy of other tree species). In each population three to five (average 4.8 ± 0.5 SD) cone bearing shrubs were randomly selected. Per shrub, three branches were randomly selected, of which on average 32.7 (± 7.7 SD) SP2 seeds and 21.7 (± 8.6 SD) SP3 seeds were sampled. In addition, from each branch, all the one year old needles were collected and pooled per shrub (cfr. the manual of ICP-forest; Rautio *et al.* 2010). The 20 populations considered here consist of a subset of the populations used in Chapter 4 for which needle nutrient concentrations were available.

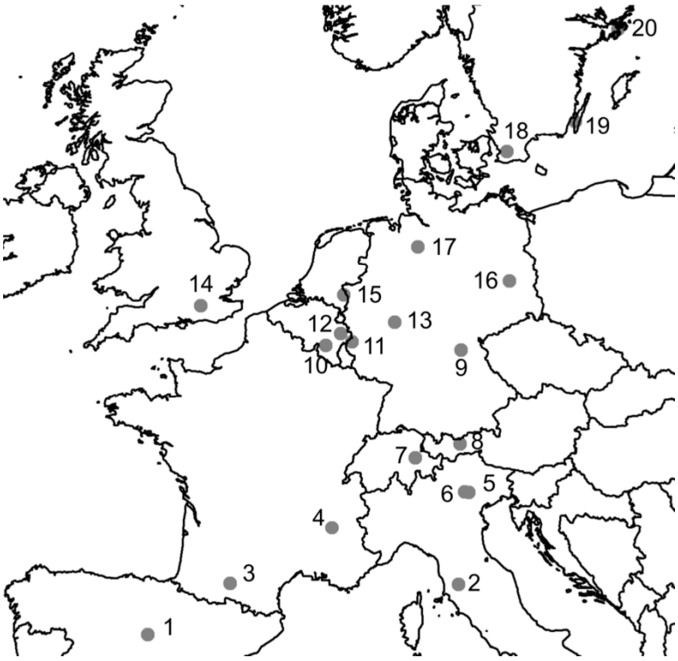


Fig. 5-1. Location and population number of the sampled common juniper populations. More information on the sampled populations is available in Appendix C1 and C2; population numbers refer to Appendix C1 and C2

5.2.2 Environmental variables

Temperature is expressed as the number of growing degree days above 0 °C base temperature ($GDD_{>0°C}$; cfr. Hall *et al.* 2002), which was calculated for the year preceding the sampling. Daily minimum and maximum temperatures of each population were obtained from the nearest weather stations (see Appendix C1 and C2) and used to calculate $GDD_{>0°C}$. When the population and the weather station had different altitudes, a mean adiabatic lapse rate correction of 5.5 $K \cdot km^{-1}$ (Körner 2007) was applied. The $GDD_{>0°C}$ ranged between 1275.1 and 5074.7 with an average of 3333.9 (± 861.9 SD).

Nitrogen and sulphur deposition data were obtained from the European Monitoring and Evaluation Programme database (EMEP)(http://www.emep.int). EMEP is the 'Co-operative Programme for Monitoring and Evaluation of the Long-range Transmission of Air pollutants in Europe' and provides scientific information on the emission, transport and deposition of air pollutants. Here, averaged data for 2006 to 2010 were used: total (wet + dry) inorganic nitrogen ($NH_x + NO_y$) depositions expressed as kg ha^{-1} $year^{-1}$ and potentially acidifying ($NH_x + NO_y + SO_x$) depositions expressed as keq ha^{-1} $year^{-1}$ in 50 × 50 km² grid cells covering Europe. Nitrogen depositions ranged from 4.85 to 28.13 kg.ha^{-1}.$year^{-1}$ with an average of 13.19 kg .ha^{-1}.year-1 (± 6.38 SD) and potentially acidifying depositions ranged from 0.51 to 2.38 keq.ha^{-1}.$year^{-1}$ with an average of 1.25 keq.ha^{-1}.$year^{-1}$ (± 0.55 SD).

Next, the average annual amount of precipitation in the five years preceding the time of sampling was calculated per population using the yearly precipitation data from EMEP. Average yearly precipitation ranged from 652.3 to 1693.4 mm.year^{-1} with an average of 978.1 mm.year^{-1} (± 295.1 SD). We used a five year average to be sure to cover the whole development period of the seed (three years) plus a short period before the start of the seed development but in which the nutrient status of the shrub can be influenced.

Finally, for each population we estimated two soil characteristics in different classes: texture of the topsoil (0-50 cm) (sandy, sandy loam, loamy, clayey) and bedrock type (calcareous vs. non-calcareous).

5.2.3 Needle analyses

Needles were dried to constant weight at 70 °C for 48 h. Concentrations of P, K, Mg and Ca were obtained after digesting 100 mg sample with 0.4 ml $HClO_4$ (65%) and 2 ml HNO_3 (70%) in Teflon pots or 4 h at 140 ºC. Phosphorus was measured colorimetrically according to the malachite green procedure (Lajtha *et al.* 1999). K, Mg and Ca concentrations were measured by atomic absorption spectrophotometry (AA240FS, Fast Sequential AAS). The concentrations of N, S and C were measured using an elemental analyzer (Vario MACRO cube CNS, Elementar, Germany). The variables that were considered for further analyses were needle concentrations of N, S, C, P, K, Ca, Mg, and ratios of C:N and N:P.

5.2.4 Seed analyses

The viability of all sampled seeds was assessed by means of stereoscopic observations of dissected seeds (3111 seeds for SP2 and 2063 seeds for SP3, in total). Again, following the method in Chapter 3, seeds that had no visible signs of anomalies were considered to have the potential to develop to the next phase and are further referred to as ‘viable seeds’.

5.2.5 Data analysis

To quantify the variation in chemical needle composition within and between populations analysis of variance was performed using the aov-function in R 2.15.1 (R Development Core Team 2012).

Linear mixed effects models using the lme-function of the nlme-library in R 2.15.1 (R Development Core Team 2012) were applied to determine the relationships between the chemical composition of the needles and environmental variables (temperature, potentially acidifying deposition, precipitation, and the interaction between temperature and depositions) (fixed effect terms). Population was added to the model as random effect term to account for the sampling within populations. Multicollinearity between temperature, potentially acidifying deposition and precipitation was verified by calculating the variance inflation factor (Quinn & Keough 2002).

For each dependent variable, all possible models (i.e. built by each combination of the selected fixed-effects terms) were compared using the Akaike's Information Criterion, adjusted for sample size (AIC_c) (Hurvich and Tsai 1989). The ΔAIC_c of a model was then calculated as the difference between the AIC_c of the model with the best fit and the AIC_c of that model. Models with $\Delta AIC_c \leq 4$ were considered equivalent (Bolker 2008). To determine the relative importance of the explanatory variables, the sum of Akaike weights of the set of all top models ($\Delta AIC_c \leq 4$) in which the variable appeared (Burnham & Anderson 2002) was used. The Akaike weight reflects the weight of evidence in support of a particular model relative to the entire model set, and varies from 0 (no support) to 1 (complete support). For each explanatory variable the relative importance was calculated by summing the Akaike weights of the models containing the variable. Finally, the averaged parameters of the top models were calculated using the model averaging function based on the AIC_c of the MuMin package in R. In addition, for each dependent variable, we calculated the r^2 for the model composed by all variables that had a importance higher than 0.60, using the following function: *r2.corr.mer<-function(m){lmfit<-lm(model.response(model.frame(m)) ~ fitted(m)); summary(lmfit)$r.squared}*..

To verify whether nitrogen deposition had a similar influence as potentially acidifying depositions, the whole procedure was repeated with nitrogen deposition instead of potentially acidifying depositions as fixed effect term.

To study the influence of the chemical composition of the needles on seed viability after seed phase two and seed phase three, the same method of model selection was used. In this case, generalized linear mixed modelling with binomial distributions was applied, using the glmmML function of the glmmML library and the lmer function of the lme4 library. This function allows to use binomial distributions since seed viability is expressed as 0 (not viable) or 1 (viable). After testing for multicollinearity between the variables that characterize the chemical composition of the needles by calculating the variance inflation factor (Quinn &

Keough 2002), only concentrations of N, S, C, P, K, Ca, Mg were selected as fixed effect terms. Again, population was added to the model as random effect term.

5.3 Results

5.3.1 Chemical composition of the needles

Needle concentrations were very variable, especially for Ca- and Mg-concentrations (Table 5-1). Although the among- and within-population variability was relatively high (Table 5-1), our values are in agreement with values found in the literature (Fig. 5-2). Average N and S concentrations were higher than values found in the literature, while Mg concentrations were lower. For K, Mg, C:N and N:P, most variability occurred between populations (Table 5-1 and Fig. 5-2). Both variability within and between populations was important for N, C, P and Ca (Table 5-1 and Fig. 5-2). Only for S, variation mostly occurred within populations (Table 5-1 and Fig. 5-2).

Table 5-1. Needle concentrations of nitrogen (N), carbon (C), sulphur (S), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and C:N and N:P ratios. . Also the population effects on nutrient concentrations are given (proportion explained is given by r²).

	Mean ($g.kg^{-1}$)	Min ($g.kg^{-1}$)	Max ($g.kg^{-1}$)	Standard deviation	Coefficient of variation	$F_{19,75}$	p-value	r²
N	14.46	8.29	21.54	2.61	18.05	5.17	< 0.001	0.57
S	1.11	0.56	1.82	0.24	21.62	1.25	0.244	0.24
C	507.03	482.52	524.87	9.8	1.93	3.73	< 0.001	0.49
P	1.22	0.69	1.92	0.28	22.95	4.24	< 0.001	0.52
K	5.76	1.66	10.84	1.58	27.43	7.99	< 0.001	0.64
Ca	10.59	1.51	25.25	4.65	43.91	9.52	< 0.001	0.55
Mg	1.44	0.53	3.02	0.50	34.72	6.67	< 0.001	0.71
C:N	36.34	23.88	61.38	7.46	20.53	7.17	< 0.001	0.70
N:P	12.19	7.31	19.28	2.15	17.64	4.91	< 0.001	0.67

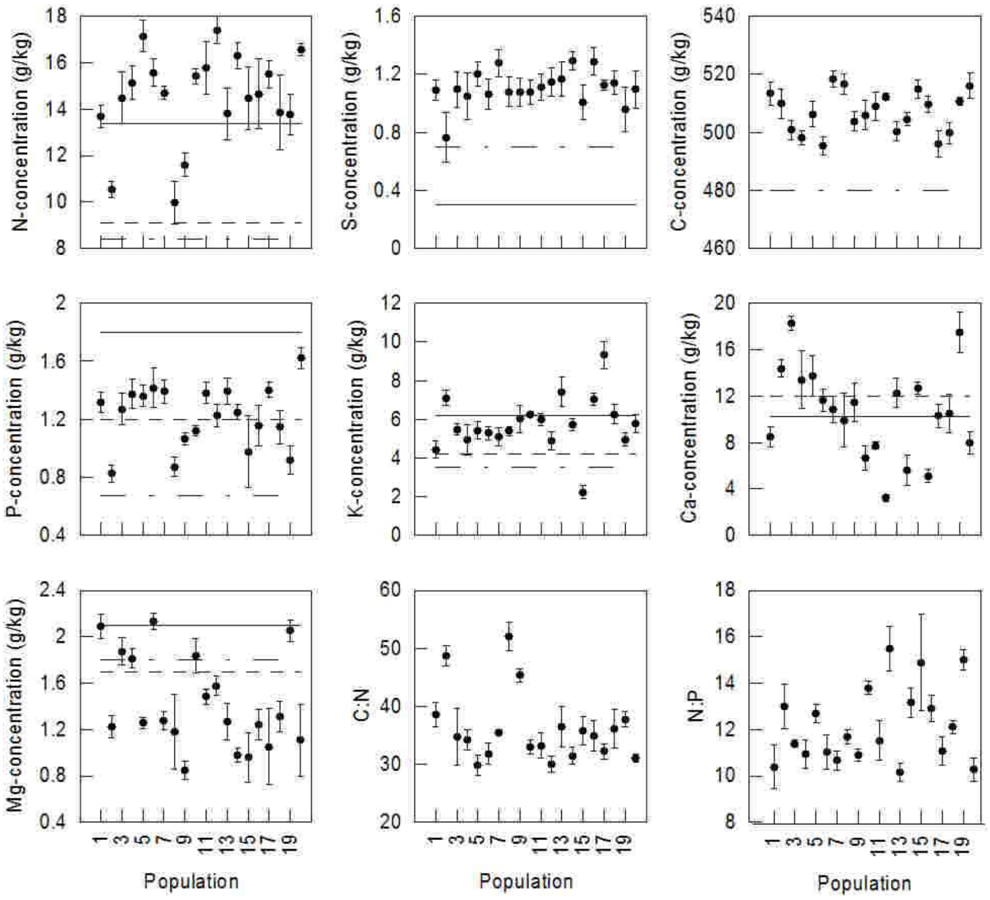


Fig. 5-2. Average needle concentrations of nitrogen (N), sulphur (S), carbon (C), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), C:N and N:P in the different populations. The full lines, the dashed lines and dotdash lines mark the average value for the specific elements found by Rodin & Bazilevich (1967), Henry (1973) and Thomas (2007), respectively. The populations are order from low to high latitude, and the numbers correspond with those of Fig. 5-1. Error bars denote standard errors.

5.3.2 Climatic and atmospheric variables vs foliar chemical composition

Temperature was strongly negatively correlated to leaf N and P and to a lesser extent to S while C:N was positively related (Table 5.2). Potentially acidifying depositions correlated negatively with leaf N, P, Ca and positively with S and C:N (Table 5.2). Interactions between temperature and potentially acidifying depositions were important for leaf N, P and C:N. Nitrogen content of the leaves was negatively correlated to potentially acidifying depositions in the cold and moderate cold regions, while in warmer regions, the relationship was positive. The correlation of potentially acidifying depositions with P was most pronounced in cold and moderately cold regions. The correlation of precipitation was positive with K and C:N and negative with N (Table 5-2). The correlations of N deposition are similar to those of potentially acidifying depositions (results not shown).

Except for S, the models could explain at least 50 % of the variation of the needle chemical composition.

Table 5-2. Importance of temperature, potentially acidifying depositions, precipitation and the interaction between temperature and potentially acidifying deposition on the needle concentrations of nitrogen (N), carbon (C), sulphur (S), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and ratios of C:N and N:P. An importance of one indicates high influence, “-“ means that the variable was not retained in any of the models.

	Temperature		Acidifying deposition		Precipitation		Temp:Acid		R^2
	Importance	Coefficient	Importance	Coefficient	Importance	Coefficient	Importance	Coefficient	
N	1	-3.29E-03	1	-4.28E+00	0.84	-1.36E-03	1	-	0.51
S	0.69	-2.09E-05	1	1.74E-01	0.27	1.05E-05	0.23	-	0.10
C	0.22	-2.25E-04	0.32	-8.02E-01	0.2	3.45E-04	-	-	0.49
P	1	-2.87E-04	0.85	-3.42E-01	0.16	-1.06E-06	0.66	-	0.49
K	0.32	6.05E-05	0.6	4.98E-01	0.65	9.93E-04	0.04	-	0.67
Ca	0.3	1.96E-04	1	-4.11E+00	0.48	1.51E-03	-	-	0.72
Mg	0.3	-2.73E-05	0.26	-2.60E-02	0.31	-7.13E-05	-	-	0.63
C:N	1	9.92E-03	1	1.16E+01	0.78	3.56E-03	1	-	0.60
N:P	0.28	9.68E-05	0.3	1.79E-01	0.37	-4.60E-04	-	-	0.56

5.3.3 Chemical composition vs seed viability

The viability of SP2-seeds was mostly correlated with leaf K concentrations (negatively) and Mg concentrations (positively). The relationships with the viability of SP3-seeds showed contrasting patterns: leaf N and S were negatively correlated with SP3-seed viability while seed viability was positively correlated with leaf P and Ca (Table 5-3).

Table 5-3. Importance of the needle concentrations of nitrogen (N), carbon (C), sulphur (S), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) for the viability of SP2- and SP3-seeds. An importance of one indicates high influence.

	SP2		SP3	
	Importance	Coefficient	Importance	Coefficient
N	0.57	-4.35E-02	1	-4.90E-01
S	0.69	-3.58E-01	0.88	-1.07E+00
C	0.21	3.30E-03	0.34	1.89E-02
P	0.22	7.35E-02	1	4.73E+00
K	1	-2.35E-01	0.66	-3.06E-01
Ca	0.68	3.47E-02	1	1.83E-01
Mg	1	5.08E-01	0.26	2.06E-01

5.4 Discussion

The aim of this research was to achieve a better understanding of the mechanisms behind the negative correlations between warming and enhanced depositions and seed viability of common juniper. Changing precipitation patterns were of minor importance for the seed viability. We assessed the potential effects of these global-change drivers on the leaf nutrient status of common juniper as a possible pathway to the negative correlations with seed viability. We first discuss how temperature and potentially acidifying depositions may affect the chemical composition of the needles. Second, we clarify how differences in the chemical composition of the needles can explain seed viability.

5.4.1 Temperature and potentially acidifying depositions vs. foliar chemistry

Leaf nutrient concentrations often reflect the nutrient availability in the soil (Aerts & Chapin 2000, Hobbie & Gough 2002). Hence, a part of the large variability in leaf nutrient concentrations between populations can be explained by differences in soil conditions. However, for most of the elements, our models explained more than 50 % of the variation. Increasing temperature correlated negatively with the concentrations of N, P and S in the needles of common juniper, and positively with the C:N-ratio. The correlations with N were only pronounced in areas with a low acidifying deposition. It is possible that an increase in relative growth rate as a response to higher temperatures is accompanied with a dilution effect on the internal N, P and S pool (e.g. Weih & Karlsson 2001, Doiron *et al.* 2014). This dilution effect theory is supported by the negative relationships between temperature and N concentration in the needles in regions with lower acidifying depositions. Acidifying depositions are strongly correlated with nitrogen deposition. Thus, in regions that are more N limited, the N pools are insufficiently amplified, to keep up with the possible higher growth rates due to warmer temperatures. Also physiological acclimation can lead to higher N- and P-concentrations in colder regions (Reich & Oleksyn 2004). For example, Hikosaka (1997) found for different plant species that optimal leaf N concentration increased with decreasing temperature.

Similar to other studies (e.g. Innes 1995; Augustin *et al.* 2005; Thimonier *et al.* 2010; Sardans *et al.* 2011), potentially acidifying depositions were positively correlated with needle N-concentrations (in the warmer regions) and S concentrations. Potentially acidifying depositions consist of N and S particles. Hence, it is not surprising that, due to higher availability, the uptake by the plants increases, leading to higher concentrations in the needles (Augustin *et al.* 2005). A possible hypothesis to explain the interaction with temperature for the N concentration is that at higher temperatures, the shrubs grow strong enough which leads to a fast N and S uptake. In colder climates, N-deposition is typically lower and competition for N, e.g. with micro-organisms, is higher. In addition, mineralisation of N is lower at colder temperatures (Rustad *et al.* 2001).

Phosphorus and Ca concentrations were negatively correlated with potentially acidifying depositions. Soil acidification, due to potentially acidifying depositions, can decrease the soil Ca-concentrations and has also an influence on the bioavailability of P in the soil. This, in turn, can influence the concentrations in the needles. For example, soil acidification also led to lower Ca-concentrations in the leaves of *Fagus crenata* (Izuta *et al.* 2004) and N deposition and acid soils negatively affected Ca- and P-concentrations in *Fagus sylvatica* leaves (Duquesnay *et al.* 2000). A decreased uptake and leaching of cations, including Ca^{2+}

caused by potentially acidifying depositions (Bobbink *et al.* 1992, Schaberg *et al.* 2001, Krupa 2003) are possible mechanisms to clarify the lower Ca concentrations in the needles.

Also the reduction of mycorrhizal associations caused by the potentially acidifying atmospheric deposition (Malcová *et al.* 1999, Krupa 2003) can help to explain the lower P-concentrations in the needles. Common juniper is mainly associated with arbuscular mycorrhiza (AM) (Thomas *et al.* 2007) and a major role of AM is to assist the infected plant with the uptake of P, especially in low P soils (Garg & Chandel 2010), where common juniper naturally occurs (Thomas *et al.* 2007).

5.4.2 Foliar chemical composition vs. seed viability

We found that needle nutrient concentrations correlated with seed viability of common juniper. Foliar nutrient concentrations can be a good proxy for the nutrient status of the whole plant (e.g. Jett 1987), and thus for the nutrient status of the seeds and their formation. For example, seed weight can be correlated to the needle N, P and K concentrations (Karlsson 2006). Seed mass was positively correlated, and the number of seeds negatively correlated, with needle N concentrations in *Pinus sylvestris* (Savonen & Saarsalmi 1999). Also in e.g. *Malus* sp., the number of flowers was positively correlated with the concentration of N, P and K in the foliage (Marschner 1995). Foliar nutrients can also have a direct effect on the development of seeds; leaf P possibly regulates resource allocation between vegetative and reproductive development (Aerts & Chapin 2000).

Needle Ca- and Mg-concentrations correlated positively with the seed viability. There are several possible explanations for this effect. For example, both elements are involved in enzyme activity (Raven *et al.* 1999) and the proportion of Ca-pectate in the cell walls is of importance for fruit ripening in plants (Marschner 1995). If the Ca concentration falls below a critical level in fast growing tissues such as fruits and storage tissues, cell wall stabilization and membrane integrity can be affected (Marschner 1995). Also, low levels of Mg can disturb the export of e.g. carbohydrates from source to sink sites in plants, as the element plays an important role in this process (Marschner 1995).

Phosphorus is not only important for plant viability but also influences seed quality and germinability (Bishnoi *et al.* 2007, Baeten *et al.* 2010). This is reflected in our results by the positive relation between the needle P-concentration and seed viability. In seeds, P is typically stored as phytate. Phytates are also the main storage sites of K, Mg and, in some cases, Ca and they are involved in the starch synthesis during seed development (Marschner 1995). Hence, P deficiency can restrict seed formation.

Both N and S concentrations were negatively correlated with seed viability and the average concentrations were lower than those found in literature (Fig. 5-2). On the one hand, their influence can also act directly. For example, detoxification, needed in case of a higher uptake of NH_4^+ and NH_3 through canopy exchange, often leads to alterations in the composition of amino acids as plants will choose to store the surplus of nitrogen in compounds with low C:N ratios (e.g. arginine) (Krupa 2003). Among amino acids, arginine is reported to be the most abundant in the female gametophyte of the conifer *Pinus banksiana* (Durzan & Chalupa 1968). Signalling in plants can also be disturbed as arginine acts as an endogenous source of stress-related nitric oxide (NO), a molecular signal that provides the signalling of adaptive structural and functional changes for survival and habituation, but also for damaging reactions, leading to cell death and necrosis (Durzan 2002). On the other hand, if N and S are sufficiently accessible to the plants, a faster growth can lead to deficiencies in other elements including Ca and Mg (Marschner 1995). As temperature correlates negatively with N concentrations, we can assume that the negative correlations between temperature and seed viability are not working through N concentrations.

5.4.3 Conclusions

The among-population variability in needle nutrient concentrations of common juniper was strikingly high. Both temperature and potentially acidifying depositions were correlated with the chemical composition of the needles. Changing nutrient availability, leaching and uptake, possibly play an important role in the altered nutrient status of the needles and, probably of the whole shrub, including the seeds. In addition, a dilution effect caused by augmented growth can also be of importance. A shortage of P, Mg and Ca, key elements during the nutrient storage throughout the seed development, can lead to anomalies and seed abortion, thereby explaining the low seed viability of common juniper in different regions throughout Europe.

6 Juniper threatened by climate warming: evidence from warming and transplant experiments along a latitudinal gradient

After Gruwez R, De Frenne P, Vander Mijnsbrugge K, Vangansbeke P, Verheyen K. Juniper threatened by climate warming: evidence from warming and transplant experiments along a latitudinal gradient. In preparation.

Abstract

Climate change is predicted to strongly affect biodiversity in the 21st century. Common juniper is rapidly becoming one of the most emblematic victims. Due to failing recruitment, its populations are decreasing in size and number in several regions in Europe. One of the main causes for this failure is low seed viability, partly induced by increasing temperatures. However, our understanding of the mechanisms behind this effect remains incomplete. Here we experimentally assess the influence of temperature on two key developmental phases during the sexual reproduction, i.e. gametogenesis and fertilization (seed phase two, SP2) and embryo development (seed phase three, SP3). At four sites along a latitudinal gradient in Europe (from South France to Central Sweden), we installed a transplant experiment with shrubs originating from Belgium. At the Belgian site we also experimentally warmed juniper shrubs using a relatively large open-top chamber. Seeds of both seed phases were sampled during three consecutive years, and seed viability was determined. Temperature negatively affected the seed viability of both SP2- and SP3-seeds along the latitudinal gradient. Interestingly, the effect on embryo development (SP3) only occurred in the third year, i.e., only when also gametogenesis and fertilization took place in warmer conditions. Our findings underscore the negative effects of increasing temperature on seed viability of common juniper. Moreover, we found strong indications that this negative influence mostly acts via disrupting the growth of the pollen tube, the development of the female gametophyte and the fertilization (SP2). This, in turn, can lead to a failing embryo development, for example, due to nutritional problems.

6.1 Introduction

The global climate system is currently undergoing one if its most rapid changes from the past 65 million years (Diffenbaugh & Field 2013). Subsequently, these changing environmental conditions are predicted to strongly affect biodiversity during the 21st century (Thomas *et al.* 2004, Warren *et al.* 2013). Species that are already stressed by other global environmental threats such as pollution, invasive species and habitat destruction and fragmentation will be particularly at risk (Thomas *et al.* 2004, Hof *et al.* 2011).

One such species, common juniper (*Juniperus communis*), is arguably one of the most iconic threatened tree species of Europe.

There is increasing evidence available that common juniper is becoming a victim of climate change. The relative lack of germinable seed production, resulting in low probabilities for successful natural regeneration, is one of the main reasons for the decline of many populations across Europe (Verheyen *et al.* 2005, Verheyen *et al.* 2009, Chapter 4). Based on observational studies using variation in climate and nitrogen deposition along latitudinal and elevational gradients, atmospheric deposition and increasing temperatures were found to negatively affect seed viability of common juniper (Verheyen *et al.* 2009, Chapter 4). However, the exact mechanisms acting during subsequent phases of the sexual reproductive cycle remain unclear. To better develop management plans of juniper populations across Europe, it is therefore essential to achieve a better understanding of the mechanisms behind the effects of warming on juniper seeds. For example, Hedhly *et al.* (2009) underlined the importance of studying the sensitive stages (e.g. fertilization, embryogenesis) independently in order to obtain a better understanding of the effect of temperature on sexual reproduction. For common juniper, the most critical phase during predispersal seed development occurs during embryo development. However, it is also possible that warming already affects seed viability during the growth of the pollen tube and female gametophyte, and fertilization (Hedhly *et al.* 2009, Chapter 3, Chapter 4).

Here we apply warming and transplant experiments along a large-scale latitudinal gradient in Europe to experimentally assess the effects of warming on viability of juniper seeds following (i) growth of the pollen tube and female gametophyte, and fertilization and (ii) embryo development. We installed transplant experiments at four sites along a latitudinal gradient in Europe (from South France to Central Sweden). The warming experiment using a relatively large open-top chamber was set up in Belgium. Seeds were sampled during three consecutive years. We specifically assessed the following hypotheses: (i) warming has a negative influence on juniper seed viability, and (ii) the negative effects of rising temperatures are most pronounced during the growth of the pollen tube and female gametophyte, and fertilization compared to effects during embryo development.

6.2 Methods

6.2.1 Experimental design

In autumn of 2011, we planted groups of nine juniper shrubs (since the species is dioecious, always consisting of two males and seven females, within a surface area of 1.2 × 1.2 m²) at four locations along a latitudinal gradient (Tyresö in Central Sweden (59.2°N, 18.3°E, 4 m above sea level, a.s.l.), Melle in Belgium, low elevation (51.0°N, 3.8°E, 15 m.a.s.l), Waimes in Belgium, relatively high elevation (50.5°N, 6.1°E, 673 m.a.s.l) and Auzeville-Tolosane in Southern France (43.5°N, 1.5°E, 170 m.a.s.l) (Fig. 6-1). In Belgium, only at the low elevation, two groups were planted. One group of shrubs was retained as control group, while the other group was experimentally warmed using an open top chamber (OTC). The rectangular OTC was 2 m high and had ground surface area of 1.5 × 1.5 m². At the top edge of the OTC, we installed an open top rectangular pyramid of 0.3 m high, with an open top area of 1.2 x 1.2 m (*cfr*. Xu *et al.* 2009; see Fig. 6-2).

Fig. 6-1. Location of the different sites in the transplant experiment (S: Central Sweden; BH: Belgium, high elevation; BL: Belgium, low elevation; F: Southern France) and of the warming experiment (BL).

Fig. 6-2. Picture of the open top chamber at Belgium low elevation.

The OTC was produced from aluminium profiles at the corners (< 3 cm wide to minimize shading) and extruded polycarbonate plates (visible and UV light transmission 86 %, IR light transmission < 5 %, DIN 5036; Quinn Plastics, Derrylin, Northern Ireland).

Mother plants growing in natural populations in Northern Belgium were vegetatively propagated by cuttings in the summer of 2008, and further grown to three year old potted plants following standard nursery practices, at the nursery of the Research Institute for Nature and Forest (50.7°N, 3.9°E). Pot size was 4 l, containing standard, commercially available soil for growing woody species. In the field sites, the shrubs were transplanted to pots of 12 l, again using standard potting soil. Hence, the pot soil will have influenced the growth in the first years after plantation (during the time of the experiments) in a similar way in the different sites, fading putative effects of heterogeneous soil conditions at these sites. Three year old plants were between 1.00 m and 1.20 m tall and most of them already in a reproductive stage. Although we made sure that clones were not planted at the same site, the shrubs were further randomly divided over the different sites, regardless of the populations of the mother plants. The genetic diversity both between and within populations is still high (Vanden Broeck *et al.* 2011) thereby probably making the origin of the mother plants less important (but see Appendix D1 for more information).

6.2.2 Seed sampling, seed analysis and shoot length

In the period of October – December of 2011, 2012 and 2013 we randomly sampled, if available, ten cones with SP2 seeds and ten cones with SP3 seeds on every female shrub at each site. The sampling of 2011 took place just before the shrubs were transplanted at the experimental sites in the nursery and can therefore be considered as a pre-treatment control. Since the shrubs were still relatively young and seed maturation takes two to three years, the amount of SP3-seeds was relatively low that year.

The viability of sampled seeds was assessed by means of stereoscopic observations of dissected seeds (2707 seeds for SP2 and 1636 seeds for SP3, in total) following the methods in Chapter 3. Seeds without visible signs of anomalies were considered to have the potential to develop to the next phase and are further referred to as 'viable seeds'. Although this method tends to overestimate seed viability inferred from tetrazolium tests, results from both methods are significantly correlated ($r = 0.681$, $n = 198$ seeds, $p < 0.01$; Adriaenssens 2006). More specifically, viable SP2 seeds contained a megagametophyte and nucellus consisting of green-white and moist tissue, not completely filling the space within the seed coat (Chapter 3). Viable SP3 seeds consisted of an embryo and megagametophyte with a smooth, white and moist surface. In this phase, almost all space within the seed coat was filled (Chapter 3). The shrubs displayed large variability in seed viability, ranging from 15.8 % to 100 % per shrub for SP2-seeds and from 0 % to 100 % per shrub for SP3-seeds (Appendix D2). The average seed viability was consistently higher for SP2 seeds compared to SP3 seeds (24 % on average over the three sampling years).

According to the trade-off theory of resource allocation, a particular period of sexual reproduction implies a certain cost to the plant. This cost can, for example, be expressed as a change in vegetative growth (Fenner & Thompson 2005). To take possible resource allocation, but also weather, ontogenetic and other effects, into account, the length of the longest, last year's shoot of each shrub was measured at the same time as the seed collection.

6.2.3 Environmental variables

The air temperature was measured during the whole experiment at two-hourly intervals in the middle of each group of shrubs (0.40 m above ground level) using Decagon Temperature probes connected to Decagon Em50 dataloggers (Decagon Devices Inc., Pullman, WA, USA). For each day, the minimum (T_{min}) and maximum (T_{max}) temperatures were calculated. The temperature in central Sweden was used as reference for easier comparison of the “warming” treatments. On average, the differences in T_{min} were + 2.1 °C (± 0.2 °C SE), + 5.7 °C (± 0.2 °C SE) and + 6.8 °C (± 0.2 °C SE) for Belgium at high elevation, Belgium at low elevation and Southern France, respectively. The average T_{max} differences were + 0.3 °C (± 0.2 °C SE), + 4.4 °C (± 0.2 °C SE) and + 8.4 °C (± 0.2 °C SE) for Belgium at high elevation, Belgium at low elevation and Southern France, respectively (see Appendix D3 for more information on monthly temperatures at the different transplant sites).

Similarly, temperatures were logged in the OTC in Belgium with Decagon Temperature probes connected to Decagon Em50 dataloggers. The average T_{min} and T_{max} differences between the plot without and the plot with experimental heating were -0.3 °C (± 0.04 °C SE) and +2.9 °C (± 0.1 °C SE) respectively.

6.2.4 Data analysis

To analyse the effects of the warming and transplant treatments per year, generalized linear modelling with binomial distributions was applied using the *glm* function in R 2.15.1 (R Development Core Team 2012). Seed viability was binomially expressed as 0 (not viable) or 1 (viable). In the transplant experiment, *site* and *shoot* were used as fixed effect terms. The sites, expressed as ordinal factors, were ordered by increasing temperature ((1) central Sweden; (2) Belgium at high elevation; (3) Belgium at low elevation; (4) Southern France) for the transplant experiment. In the warming experiment, site was replaced by the experimental temperature treatment ((0) no warming; (1) warming).

To study the differences between the sites in the transplant experiment, Tukey's honestly significant difference tests were performed, using the *glht* function of the *multcomp* library.

6.3 Results

6.3.1 Transplant experiment

Before transplantation, there were only minor differences in seed viability between the shrubs that were assigned to the different sites (Table 6-1). However, already after one year, shrubs that were growing in colder sites had a higher SP2-seed viability (Table 6-1; Fig. 6-3). It is remarkable that only for the sites with the largest differences in temperature, the differences were highly significant. Seeds of shrubs transplanted between sites with smaller temperature differences displayed no or only marginal viability differences, especially when taking into account the differences before transplantation.

Table 6-1. The effects (parameter estimates) of the transplant site and warming treatment on viability of seed phase 2 seeds and seed phase 3 seeds for the different years of sampling. Shoot length was included as a covariate. For the transplant experiment, the effects of temperature were assessed by comparing seed viability between the different sites (S: Central Sweden; BH: Belgium, high elevation; BL: Belgium, low elevation; F: Southern France).

			Seed phase 2 seeds			Seed phase 3 seeds		
			Before transplantation	After transplantation		Before transplantation	After transplantation	
			2011	2012	2013	2011	2012	2013
Transplant experiment	Site	BH-S	-0.43	-0.56	0.19	0.29	-0.36	-0.68 *
		BL-S	-1.14 *	-1.05 *	-0.89 **	-1.45	-0.7	-1.89 ***
		F-S	-0.25	-1.52 ***	-1.64 ***	-0.67	-0.88	-5.63 ***
		BL-BH	-0.71	-0.49	-1.08 ***	-1.74 *	-0.34	-1.21 ***
		F-BH	0.18	-0.96 *	-1.82 ***	-0.96	-0.52	-4.95 ***
		F-BL	0.90	-0.47	-0.75 ***	0.78	-0.18	-3.74 ***
	Shoot length		-0.03**	0.04	0.05*	-0.04 ***	0.03	0.04*
Warming experiment	Warming		1.67 **	-1.3 **	-0.23	0.58	0.69	1.2 ***
	Shoot length		-0.01	0.09 **	0.03	-0.03	0.07 **	0.06 **

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

In 2013, the differences between sites were highly significant for both SP2- and SP3-seeds, with more viable seeds at colder sites, except between the sites in central Sweden and at the higher elevation in Belgium. There was no or only marginal covariation between the length of the shoots and seed viability, except for both seed phases in 2011 (negatively).

Based on Appendix D3 we also found that the differences in average temperatures between 2012 and 2013 were only for Belgium high elevation higher than 1.0 °C (i.e. 7.4 vs. 6.3 °C). However, the seed viability at that site did not changed significantly over these two years (Fig. 6-3).

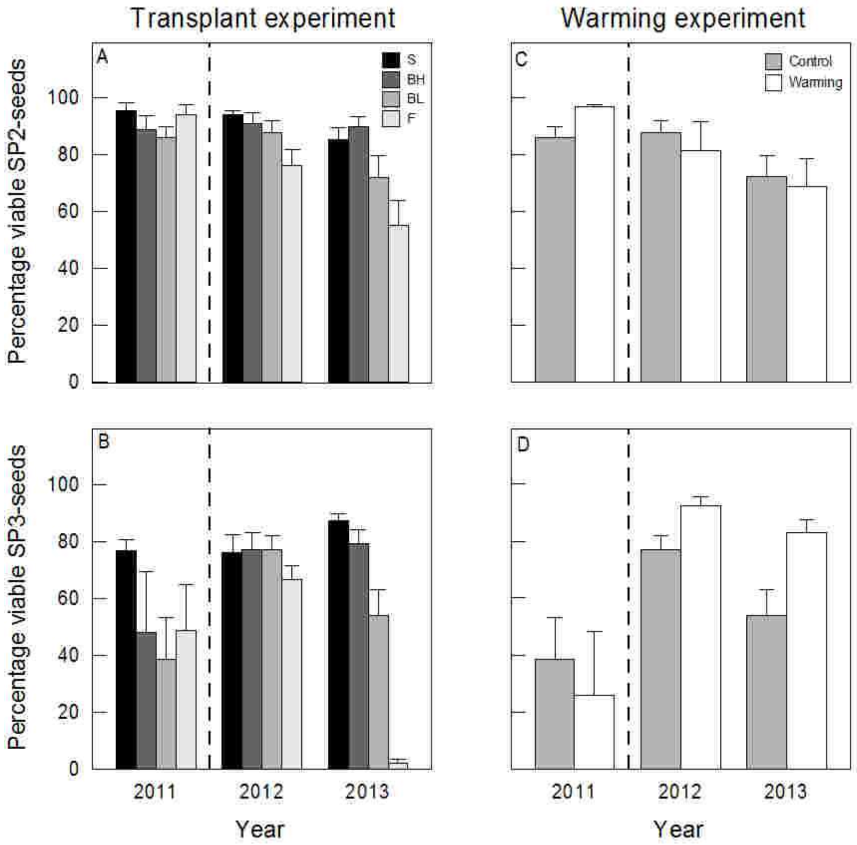


Fig. 6-3. Viability of juniper phase 2 (SP2) seeds (A and C) and phase 3 (SP3) seeds (B and D) in the transplant and warming experiments (in percentage viable seeds per shrub). Bars at the left of the dotted lines show pre-treatment values. Error bars represent standard errors (S: Central Sweden; BH: Belgium, high elevation; BL: Belgium, low elevation; F: Southern France)

6.3.2 Warming experiment

In 2011, shrubs planted in the open top chamber had a larger amount of viable SP2-seeds than the shrubs planted in ambient air (Table 6-1; Fig. 6-3). Even though an initial higher percentage of viable SP2 seeds in the warming plots was present before the experimental treatments started, the SP2 viability decreased after one and two years of warming. In 2012, the opposite was true for SP3 seeds. Shoot length had a positive influence on seed viability for both SP2- and SP3-seed in 2012 and only for SP3-seeds in 2013.

6.4 Discussion

By applying an unusual combination of warming and transplant experiments across Europe, we were able to experimentally assess the effects of warming on seed viability of common juniper. In the transplant experiment, the negative influence of increasing temperatures from north to south was already visible after one year for the SP2-seeds. After two years, the effect was even more pronounced. Viability of SP3-seeds was only negatively affected after two years. During seed phase two, higher temperatures can directly induce abnormalities in the female gametophyte (Franz & Jolliff 1989, Kozai *et al.* 2004) and other female structures (Saini *et al.* 1983, Hedhly *et al.* 2003). In addition, it is hypothesized that the ovule and female gametophyte might be important regulators of pollen tube growth (e.g. Fernando *et al.* 2005, Drews & Koltunow 2011). This is thus a possible pathway of an indirect effect of temperature on seed viability. Finally, via different influences on the growth of the pollen tube and female gametophyte, high temperatures can also have detrimental effects on the male-female synchrony in the pre-fertilization phases (Zinn *et al.* 2010, Hedhly 2011).

During embryo development (SP3), higher temperatures may disrupt meristematic activity of the megagametophyte with accumulation of resources such as lipids, starch and proteins (Singh 1978, Owens *et al.* 2008) and the nutrition and growth of the embryo. However, the effect of increasing temperatures on SP3-seeds only occurred when gametophyte development, fertilization and early embryo development had taken place under warmer conditions and this effect on SP3-seeds in 2013 was more pronounced than the influence of temperature on SP2-seed viability in 2012. This strongly suggests that abortions during embryo development can have their cause in seed phase two. For example, anomalies during the growth of the female gametophyte can lead to a badly developed megagametophyte, which in turn can disturb the nourishment of the developing embryo during seed phase three.

In the transplant experiment, except for SP3-seeds in 2011, we found no indications that vegetative growth was correlated with seed viability. Assuming that shoot growth correlates with resource allocation, this indicates that the allocation of resources to vegetative growth or sexual reproduction is of minor importance for common juniper. Furthermore, the correlation between shoot length and the SP3-seed viability in 2011 should be assessed with caution as the amount of sampled seeds was very low.

The results of the OTC-experiment were less equivocal. First, there was already a significant difference in the viability of SP2-seeds in 2011, before the experiment had started. Second, we found a negative relationship with temperature for the viability of SP2-seeds in 2012 and a positive relationship for SP3-seeds in 2013. Also shoot length correlated positively with seed viability in different years. In addition, the differences in temperature were much smaller compared with the differences measured in the transplant experiment. An important drawback of this method is also that other abiotic and biotic factors such as precipitation, relative air humidity, herbivory or pollination can be affected by the enclosed design of OTCs such that the temperature signal is confounded with changes in other environmental variables (De Frenne *et al.* 2010). We are also aware that the fact that we had no replications of the open-top chamber is a major drawback. However, there were only a limited amount of male and female shrubs available, so a larger experimental design was not possible. Hence, especially the results of the OTC-experiment should be assessed within this context.

In this experiment we found no indications that common juniper in Belgium demonstrates local adaptation concerning seed viability. Shrubs that were planted in Belgium did not perform better than shrubs that were transplanted farther away. However, this conclusion can not automatically be generatized for the region of this experiment as we did not transplant shrubs from e.g. Sweden or France.

The average seed viability in this experiment was much higher compared to seed viability of juniper recorded across Europe (e.g. SP3 seed viability of < 3 % in Chapter 3, compared to an average of 60 % in this experiment). The reasons for this higher seed viability are not completely clear. It is possible that younger and more vital shrubs have a higher chance of producing viable seeds. The age of the cuttings at the start of our experiment was about three years. In addition, during those three years, the shrubs grew in optimal conditions, in nutrient rich standard potting soil for woody species. Common juniper typically occurs on nutrient poor soils (both acidic and calcareous) and relatively harsh environments such as in heathlands and alpine ecosystems (García *et al.* 2000, Thomas *et al.* 2007, Ward 2007). Yet, fertilization experiments had no influence on the survival and growth of juniper plants (Thomas *et al.* 2007). However, shortage of soil phosphor, calcium and magnesium can have negative influence on the seed viability (Chapter 5). Hence, it is still possible that common juniper prefers richer soils but is outcompeted by other species on these sites (Pearman *et al.* 2008).

The results of this research deliver strong indications that increasing temperatures decrease the seed viability of common juniper, particularly by disrupting the growth of the pollen tube and female gametophyte, and fertilization. Since the negative effects of increasing temperature on embryo development (SP3) only occur when the growth of the male and female gametophytes, fertilization and early embryo development (SP2) took place in warmer conditions, this enhances the hypothesis that the processes during seed phase two are most vulnerable for increased temperatures. However, the relatively high seed viability that was measured in this experiment compared to evidence of low seed viability in the European lowlands (Chapter 4) shows that, besides temperature, also other factors such as soil conditions and shrub age can influence seed viability. Hence, as climate warming seems inevitable in the coming decades, more research on how seed viability and regeneration of common juniper can be increased is needed to preserve this threatened species. Nevertheless, the findings of this study can help to soundly inform policy and management decisions regarding the conservation of juniper communities. For instance, management of common juniper could be focussed on populations with colder microclimates (for example north facing slopes (Scherrer & Körner 2010) and the vicinity of small rivers (Fridley 2009)) or on populations that grow on richer soils. Also increasing the number of young individuals in a population with shrubs originating from cuttings can give an extra input of viable seeds.

7 Effects of management actions on the recruitment of threatened common juniper populations (*Juniperus communis*)

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Abstract

Common juniper is one of the most wide spread plant species. However, the last decades juniper populations are decreasing in size and number in different regions. Lack of recruitment, caused by extreme low seed viability and the absence of suitable microsites for recruitment, is the key reason for this decline.

For successful germination, the seeds need gaps in the existing vegetation and a soil with a relatively high base saturation degree. Sod cutting, rotavation and liming are three management actions in heathlands that have potential to achieve these conditions. However, their actual effects on seed germination and seedling survival of common juniper are still not known. Here we installed in four different sites in Belgium and the Netherlands a sowing experiment to assess how these management actions (alone or in various combinations) influence soil characteristics and recruitment of common juniper. We also determined the preferred soil conditions for improved seed germination and seedling survival of common juniper.

Our results corroborate that bare ground is preferable for germination of common juniper. Secondly, low silt and lutum portions in the soil and low soil organic matter content (two soil characteristics that can be linked with drought stress) seemed to have a negative impact. Actions that lower those characteristics, as deep sod cutting, should be avoided at drought-stressed locations. When both restrictions are met, the soil pH is an important characteristic to determine whether liming is a possibility or not, although this measure should be taken with caution on nutrient poor soils.

7.1 Introduction

Together with European yew (*Taxus baccata*) and Scots pine (*Pinus sylvestris*), common juniper is the only native coniferous tree species in Belgium and the Netherlands. In these regions, the species mainly occurs on nutrient poor, acid, sandy soils, typical for heaths and drift sands. Due to land-use changes related to afforestation, agriculture and urbanisation, the area of heathland and drift sands has largely declined in the 20th century (Webb 2002, Piessens *et al.* 2004, Piessens *et al.* 2005). In addition, increased atmospheric deposition of nitrogen and sulphur caused soil acidification in the remaining heathlands and disturbed the nutrient balance (Bobbink *et al.* 1998). Despite the efforts during the latest decades for stopping habitat destruction and degradation, the decline of common juniper is still on-going in these regions, mainly because of a lack of recruitment (e.g. Verheyen *et al.* 2009). Verheyen *et al.* (2009) revealed a triangular relationship between the fraction of recently recruited individuals and the percentage viable seeds in a population. This means that if seed viability is low, recruitment is negligible, while in case of a high percentage of viable seeds other factors such as herbivory, summer drought and the absence of suitable microsites for germination are responsible for the differences in recruitment between populations. Thus, not only low seed viability causes the lack of recruitment (Ward, 1973, Fitter & Jennings 1975, Gilbert 1980, Ward 1982, García 2001).

For successful recruitment, the seeds need gaps in the existing vegetation and soil with a relatively high base saturation degree. In addition, seeds should be covered by a thin layer of soil, kept relatively moist and germinate in open habitats, free of shade-casting vegetation (McVean 1966, Livingston 1972, Ward 1973, Fitter & Jennings 1975, Clifton *et al.* 1997, Hommel 2009). Although grazing by e.g. sheep and cattle can help to achieve these conditions (e.g. Fitter & Jennings 1975, Hommel 2009), this management also risks eliminating new seedlings and damaging the mature shrubs by grazing (Ward 1973, Clifton *et al.* 1997). Taking this risk into account and the already threatened status of the remaining *J. communis* populations in Belgium and the Netherlands, there is a need for other soil treatments to obtain suitable microsites for germination.

Removal of organic material by sod cutting such that gaps are created and competition of other plants reduced is an often used management technique to maintain oligotrophic systems such as heathlands (Aerts & Heil 1993). However, due to the on-going acidification of heathlands in Belgium and the Netherlands, the soil pH is shifting towards the aluminium and iron buffer range resulting in elevated soil Al^{3+} availability ($pH < 4.5$; Bowman *et al.* 2008). High soil Al^{3+} concentrations can be toxic for plants and prevent germination of seeds (Ulrich & Sumner 1991; van den Berg *et al.* 2003). The toxicity of Al^{3+} can be reduced if the Al^{3+} is complexed with organic compounds (Ulrich & Sumner 1991). However, through sod cutting, the organic matter concentration in the soil and the reduction potential of Al^{3+} may decrease.

Additional liming to move the soil in the cation exchange buffer range (8 > pH(H_2O) > 4.5; Bowman *et al.* 2008) can therefore be useful (van den Berg *et al.* 2003, Dorland *et al.* 2004). Finally, rotavation, i.e. mechanically breaking up the soil, can also create bare ground. As a pronounced pH gradient exists in sandy heathland soils (De Bakker 1979), rotavation can also increase soil pH due to mixture with lower, less acid soil. However, this management can negatively influence other species as their seed bank can get buried too deep.

Although the above mentioned management actions have potential for ameliorating the conditions for germination and seedling survival of common juniper, the actual effects and their mechanisms are still not known.

Here we experimentally assess how management actions in heathlands including sod cutting, rotavation, and liming (alone or in various combinations) influence soil characteristics and determined the preferred soil conditions for improved common juniper seed germination and seedling survival. Finally, we studied the direct effects of the different management actions in heath on the recruitment of common juniper.

7.2 Methods

7.2.1 Experimental design

Two similar experiments to examine the influence of different soil treatments on the recruitment of common juniper were conducted in Belgium and the Netherlands.

In Belgium, the experiment was installed during the winter of 2008-2009, two months before sowing. We installed 24 1 × 1 m² experimental plots at two different sites in the Campine region (Mechelse Heide (N51.0°, E5.6°, 95 m.a.s.l.) and Ten Haagdoorn Heide (N51.0°, E5.4°, 72 m.a.s.l.)). The plots were installed in 4 × 6 rows. Treatments 3 to 6 (see further) were randomly mixed in the first four rows. The two other treatments (without sod cutting; see further) were in the last two rows. There was a distance of 0.5 m between each plot (except between row four and five, where the distance was 1.0 m). Both sites are dry heathlands on a poor, loamy (11.3 % loam at Mechelse Heide and 9.0 % at Ten Haagdoorn Heide) sandy soil dominated by *Calluna vulgaris* shrubs of approximately 0.5 m high. At each site six treatments were applied (4 replicates per treatment and per site): (1) control (no treatment (C)); (2) fencing (F); (3) fencing + deep sod cutting (F+S); (4) fencing + deep sod cutting + lime addition (F+S+L); (5) fencing + deep sod cutting + rotavation (F+S+R); (6)

fencing + deep sod cutting + lime addition + rotavation (F+S+R+L). Fencing (1.5 m high, 0.05 m mesh width, and until 0.2 m depth in the soil) excluded small (e.g. rabbits) and large (e.g. roe deer) mammalian herbivores. In the sod cutting treatment, we removed the organic layer while in the ‘rotavation’ treatment, the soil was turned over and mixed until a depth of 0.3 m. In the lime addition treatment, 2000 kg ha^{-1} dolomitic lime (48 % CaO + MgO; 33 % CaO; 15 % MgO; max. 6 mm granules) was added to the plots.

We used seeds of four different provenances to also determine the effects of seed origin and seed viability. We sampled seeds in Belgium (As (N51.0°, E5.6°)), but also in populations from regions in Europe where seed viability is still relatively high (Ekulunde (N56.6°, E16.6°) in Sweden, Kleszczele (N52.6°, E23.3°) in Poland, Rossdach (N50.0°, E11.1°) in Germany) (see Chapter 3 for more information on these juniper populations). During November and December of 2008, ripe cones were sampled in each of the populations on 10 to 15 randomly selected, cone bearing shrubs. During the winter of 2008-2009, the cones were stored outdoors, shaded and dry in open plastic pots. Every plot was divided into four subplots of 0.4 × 0.4 m². In early February we sowed the cones in these subplots (25, 20, 30 and 30 seeds per subplot for the Ekulunde, Kleszczele, Rossdach and As populations, respectively) and, where sod cutting was applied, covered them with a thin layer of soil. We also avoided a buffer zone of 0.1 m around each subplot to exclude edge effects. Germination (i.e. if the first needles were visible) and survival of the seedlings (i.e. the needles remain green) was recorded in autumn of 2009, spring and autumn of 2010 and summer and autumn of 2011. For each population, the average amount of seeds per cone and the percentage of potentially viable seeds was determined by opening a subset of 50 cones per population, counting the seeds in the cones and cutting the seeds. If the embryo and megagametophyte were white and smooth, the seeds were considered as potentially viable (see Chapter 3 for a detailed account of the methods). Seed viability determination happened in winter of 2008.

Second, in the Netherlands, the experiment was also installed on two locations: Mantinge (N52.8°, E6.6°, 50 m.a.s.l.) and Markelo (N52.3°, E6.5°, 50 m.a.s.l.). The soil of the first site developed in drift sand and has a very low loam content (1 %). The second location has a soil that developed in cover sand with a loam content of ca. 7 %). Each experimental site existed of 28 plots of 1.5 × 1.5 m² of which the central part (1 × 1 m²) was used to sow the seeds and the exterior for the soil sampling. Seven different treatments were applied to the plots: (1) fencing (F)); (2) fencing + shallow sod cutting (F+S1); (3) fencing + deep sod cutting (F+S2); (4) fencing + deep sod cutting + litter addition (F+S2+Li); (5) fencing + deep sod cutting + rotavation (F+S2+R); (6) no fence + deep sod cutting + lime addition (S2+L); (7) fencing + deep sod cutting + lime addition (F+S2+L). In the shallow sod cutting treatment, the sod was removed until a depth of c. 2 cm, while in the deep sod cutting treatment the sod and part of the mineral soil rich in organic matter until a depth of c. 5 cm was taken away. In plots with litter addition, a layer of 5 cm thick common juniper litter was spread over the plot. Similar to the Belgian experiment, rotavation turned over and mixed the soil until a depth of 0.3 m. Liming was performed by spreading 2000 kg ha^{-1} dolomitic lime (84 % CaCO3; 10 % MgCO3). The fencing again excluded herbivores such as rabbits and roe deer (same type of fences as in Belgium). The plots were divided in two equally-sized subplots. In the first subplot, c. 1000 cones per subplot were sown between February and March 2008. The cones were sampled in the autumn and winter of 2006-2007 in three areas in the Netherlands (Dwingelderveld (N 52.8 °, E6.4°), Mantinge (N52.8°, E6.6°) and Junner Koeland (N52.5°, E6.5°)). The average amount of seeds per cone and the percentage viable seeds were estimated using the data of the Dutch populations sampled Chapter 3. In the second half of the subplots, c. 800 cones per subplot were sown in March 2009. These cones were sampled in November 2008 in a common juniper population, Buinen (N52.9°, E6.8°) in the Netherlands, which still exhibits high levels of recruitment (up to 70 seedlings/m²). Similarly to the Belgian experiment, a subsample of 50 cones was used to calculate the average amount of seeds per cone and the viability. However, this subsample was only studied after the sowing event.

During both sowing events the cones were placed in four rows and covered with c. 0.5 cm sand, except in the reference plots (F), where the cones were sown randomly and not covered.

Germination and survival of the seedlings was recorded in the spring, summer and autumn of 2008 and 2009, in the summer and autumn of 2010 and in the autumn of 2011.

The movement of the cones, caused by the wind and rain, made it hard to determine with certainty whether a seedling was originating from seeds of the first or the second sowing event. Therefore we analysed germination and seedling survival of the two subplots together and used the average of both sowing events for the amount of seeds per cone and the seed viability.

7.2.2 Soil sampling and analysis

In each subplot of the Belgian experiment, 4 soil samples (0-10 cm) were collected during winter 2011-2012, pooled per plot, dried at 40 °C for 48 h, and sieved through a 1 mm mesh. In the plots where there was no sod cutting, the humus layer was removed before soil samples were taken. The soil pH-KCl of each sample was analysed using a glass electrode (Orion, Orion Europe, Cambridge, England, model 920A) after extracting 14 mL soil in a 70 mL KCl (1 M) solution. The percentage of silt + lutum (lutum is the part of the soil consisting of particles $\leq 2\ \mu$m) in the soil was estimated using procedures of the Dutch Soil Survey Institute (Conform Soil Survey Staff (1975)). Soil organic matter was estimated by loss on ignition (four hours at increasing temperature until 450 °C) as soil organic matter = 100 - % of ashes residue. Total phosphorus (P) was colourimetrically analysed following the method of Scheel (1936).

NH_4^+-acetate- EDTA extractable K^+, Mg^{2+}, Ca^{2+} and Al^{3+} concentrations were analysed by atomic absorption spectrophotometry (AA220, Agilent Technologies Belgium, Diegem, Belgium) after shaking 10 g of dry soil in 50 mL NH_4^+-acetate-EDTA solution (192.5 g NH_4^+-acetate, 50 mL acetic acid, and 29.225 g EDTA, diluted to 2 L) for 30 min.

In the Netherlands, in each plot the humus layer was described and four soil samples (0-10 cm) were collected, pooled, air dried and sieved through a 0.5 mm mesh. If present, the litter layer was removed before sampling. Moisture was determined after drying the sieved samples at 105 °C for 4 h. The weight of the air dried soil was corrected for its moisture content. We determined pH-KCl and the percentage of silt + lutum in the soil similarly as for the sites in Belgium. Soil organic matter was estimated by loss on ignition (at 380 °C) as soil organic matter = 100 - % of ashes residue Total phosphorus (P) was analysed using a Kjeldahl-destruction). Exchangeable Ca^{2+}, Mg^{2+}, K^+ and Na^+ was analysed by atomic absorption spectrophotometry after a Bascomb extraction at pH = 8.1 (Bascomb 1964).

7.2.3 Data analysis

To calculate the effects of the different treatments on the soil characteristics, the Belgian and Dutch data were analysed separately. For both experiments, linear mixed-effects models with the *lmer* function of the *lme4* library in R 2.15.1 (R Development Core Team 2012) were applied and *site of the experiment* was added as random-effect. To fulfil normality and homoscedasticity assumptions, logarithmic transformations were performed on soil organic matter, silt + lutum-content, pH, P, Mg^{2+}, Ca^{2+} and Al^{3+} for the Belgian experiment and on soil organic matter, silt + lutum-content, Mg^{2+} and Ca^{2+} for the Dutch experiment.

To analyse the influence of three soil characteristics (soil organic matter (OM), silt + lutum content and pH), fencing and rotavation on germination and mortality of juniper seedlings, linear mixed-effects models with the *glm* function (if no random effect factor was added) on the one hand, and *lmer* function of the *lme4* library on the other hand, in R 2.15.1 (R Development Core Team 2012) were applied. First, the proportion of potentially viable seeds that germinated at the end of 2011 and the proportion of seedlings that survived by the end of 2011 were calculated per plot. OM, silt + lutum content, pH, rotavation and fencing were included as fixed effect factors. To control whether the effects of OM, silt + lutum content and pH were due to differences between or within locations and due to physical differences caused by deep sod cutting, four different models were run: (1) without random effect factors; (2) with *site* of the experiment as random effect factor; (3) with *deep sod cutting* as random effect factor, and (4) with *site* of the experiment and *deep sod cutting* as random effect factor. The models were compared by calculating the Akaike's Information Criterion (AIC) for each model (Hurvich & Tsai, 1989): models with the lowest AIC were retained since these have the best fit. Due to different measuring methods in the two experiments, other soil characteristics could not be included in this analysis.

In the final step, the Belgian and Dutch data were again analysed separately. We calculated the proportion of seeds that germinated at the end of 2011 per plot for the Dutch data and per subplot for the Belgian data.

The effects of the soil treatments on the portion of germinated seeds were analysed with linear mixed-effect models, using the *lme*-function. *Site* of the experiment and *origin* of the sown seeds were added to the models as random-effects for the Belgian experiment and only site of the experiment for the Dutch experiment. To meet normality and homoscedasticity assumptions, germination proportion was first arcsine square-root-transformed. The same method was used for mortality, where we calculated the proportion of seedlings that died at the end of 2011.

7.3 Results

7.3.1 Germination rates vs. origin

We counted only 176 seedlings for ca. 260,491 sown seeds across both countries. Thus, average germination rates were very low, especially for seeds that originated from Belgium and the Netherlands (Table 7-1). The Belgian seeds displayed extremely low seed viability. The Rossdach population (Germany) delivered the most viable seeds. Absolute germination rates differed with a factor of ca. 50, while germination rates of the viable seeds were less different between the origins (a factor of ca. 17). In the latter case, germination rates in the Dutch experiments were still very low.

Table 7-1. Information on the number of sown seeds, their viability and the percentage of germinated seeds per origin.

Origin	Number of cones sown	Average seeds/cone	Number of sown seeds	Percentage viable seeds	Number of seedlings across all plots	Percentage seeds germinated	Percentage viable seeds germinated
Ekulunde (Sweden)	1200	2.91	3492	3.87	7	0.20	5.18
Kleszczele (Poland)	960	2.57	2467.2	4.32	18	0.73	16.89
Rossdach (Germany)	1440	2.62	3772.8	26.62	66	1.75	6.57
As (Belgium)	1440	2.58	3715.2	0.65	2	0.05	8.28
The Netherlands	86400	2.86	247044.2	3.35	83	0.03	1.00

7.3.2 Soil characteristics vs. treatment

In Belgium, the effects of liming (treatments F+S+L and F+S+R+L) on the soil are very clear for pH and the concentrations of Mg^{2+}, Ca^{2+} and Al^{3+} (Fig. 7-1 G, H, K, L). In both treatments pH, Mg^{2+}-concentration and Ca^{2+}-concentration were significantly higher than in the control plots and Al^{3+}-concentrations were significantly lower (Table 7-2). Total P-content was in all treatments were sod cutting took place significantly lower (Table 7-2).

The Dutch experiment showed significant increases of pH in all treatments (Table 7-2) except in the plots where only shallow sod cutting (F+S1) took place. Soil organic matter strongly decreased in treatments with deep sod cutting and also in the treatment with deep sod cutting and litter additions. Mg^{2+}- and Ca^{2+}-concentrations were significantly lower in plots with treatment F+S2+R and with treatment F+S2 and higher in the plots with treatment S2+L

(Table 7-2). The percentage silt + lutum was remarkably lower in Mantinge and in treatments F+S2, S2+L and F+S2+L (Table 7-2), the percentages significantly decreased compared with the control (F). Total P-content was in all treatments were sod cutting took place significantly lower except for the rotavation plots (Table 7-2).

Table 7-2. Soil variables in the different treatments compared to the control. The direction of the arrows indicates a higher (↑) or lower (↓) value in the treatment than in the control. The number of arrows reflect the significance of the statistical test.

Country	Treatment	Soil variables						
Belgium		Log(OM)	Log(silt + lutum)	Log(pH)	Log(P)	Log(Mg^{2+})	Log(Ca^{2+})	Log(Al^{3+})
	F	n.s.	n.s.	n.s.	n.s.	n.s.	↑↑	n.s.
	F+S	↓	n.s.	n.s.	↓↓↓	n.s.	n.s.	n.s.
	F+S+L	↓	n.s.	↑↑↑	↓↓	↑↑↑	↑↑↑	↓↓↓
	F+S+R	↓	n.s.	n.s.	↓↓↓	n.s.	n.s.	n.s.
	F+S+R+L	n.s.	n.s.	↑↑↑	↓↓	↑↑↑	↑↑↑	↓↓↓
the Netherlands		Log(OM)	Log(silt + lutum)	pH	Log(P)	Log(Mg^{2+})	Log(Ca^{2+})	
	F+S1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	
	F+S2	↓↓↓	↓↓	↑↑↑	↓	↓↓↓	↓↓↓	
	F+S2+Li	↓↓↓	n.s.	↑↑↑	↓↓	n.s.	n.s.	
	F+S2+R	n.s.	n.s.	↑↑↑	n.s.	↓	↓	
	S2+L	↓↓↓	↓↓	↑↑↑	↓↓	↑↑	↑	
	F+S2+L	↓↓↓	↓	↑↑↑	↓	n.s.	n.s.	

n.s.: not significant, ↓ or ↑: $p < 0.05$, ↓↓ or ↑↑: $p < 0.01$, ↓↓↓ or ↑↑↑: $p < 0.001$, F: fencing; S: deep sod cutting in Belgium; S1: shallow sod cutting; S2: deep sod cutting in the Netherlands; L: liming; R: rotavation; Li: addition of common juniper litter; OM: soil organic matter.

7.3.3 Germination and mortality vs. soil characteristics

The amount of organic matter in the soil had a significant positive influence on the germination in all models except when only site was used as a random-effect factor (Table 7-3). This suggests that the effect is partly due to overall differences in soil organic matter between the different sites (Fig. 7-1 C, D, O, P). However, there is still a significant effect within the sites and within plots with or without deep sod cutting. The silt + lutum content only had a significantly positive effect if site was not used as a random effect factor (Table 7-3), thus it is mostly caused by the different overall silt + lutum contents of the four sites (Fig. 7-1 E, F, Q, R). There is a significant positive relationship between pH and germination for all models except if site + deep sod cutting are random effect factors (Table 7-3).

The positive pH effect persisted, even if site or deep sod cutting was used as random effect factor. Hence, subtle differences in pH between plots in the same location or in the same level of sod cutting, can have an influence on germination.

Table 7-3. The effects (estimates) of soil organic matter, silt + lutum content, pH, rotavation and fencing on the germination success and mortality calculated by using four models: (1) without random effect factors; (2) with site as random effect factor; (3) with deep sod cutting as random effect factor, and (4) with site and deep sod cutting as random effect factor.

		No random effect factor	Site	Deep sod cutting	Site + deep sod cutting
Germination success	Soil organic matter	0.26***	n.s.	0.32***	0.31**
	Silt + lutum content	0.27***	n.s.	0.28***	n.s.
	pH	0.97***	0.65***	0.56**	n.s.
	Rotavation	n.s.	n.s.	n.s.	n.s.
	Fence	0.94***	n.s.	0.86***	0.69*
Mortality	Soil organic matter	n.s.	n.s.	n.s.	n.s.
	Silt + lutum content	n.s.	n.s.	n.s.	n.s.
	pH	n.s.	n.s.	n.s.	n.s.
	Rotavation	n.s.	n.s.	n.s.	n.s.
	Fence	n.s.	n.s.	n.s.	n.s.

**: $p < 0.01$
***: $p < 0.001$

The model with site + deep sod cutting as random effect had the lowest AIC (234 vs. 493 for the model without random effects, 294 for the model with location as random effect and 336 for the model with deep sod cutting as random effect). In this model, only the amount of soil organic matter has a significant positive influence on germination (Table 7-3). Based on the data, fencing had a positive effect on germination. However, it is more likely that some seedlings that germinated outsite the fence were immediately consumed by herbivores before they could be counted. Hence, this is probably an artefact that explaines more about mortality than about germination.

None of the variables had a significant effect on mortality.

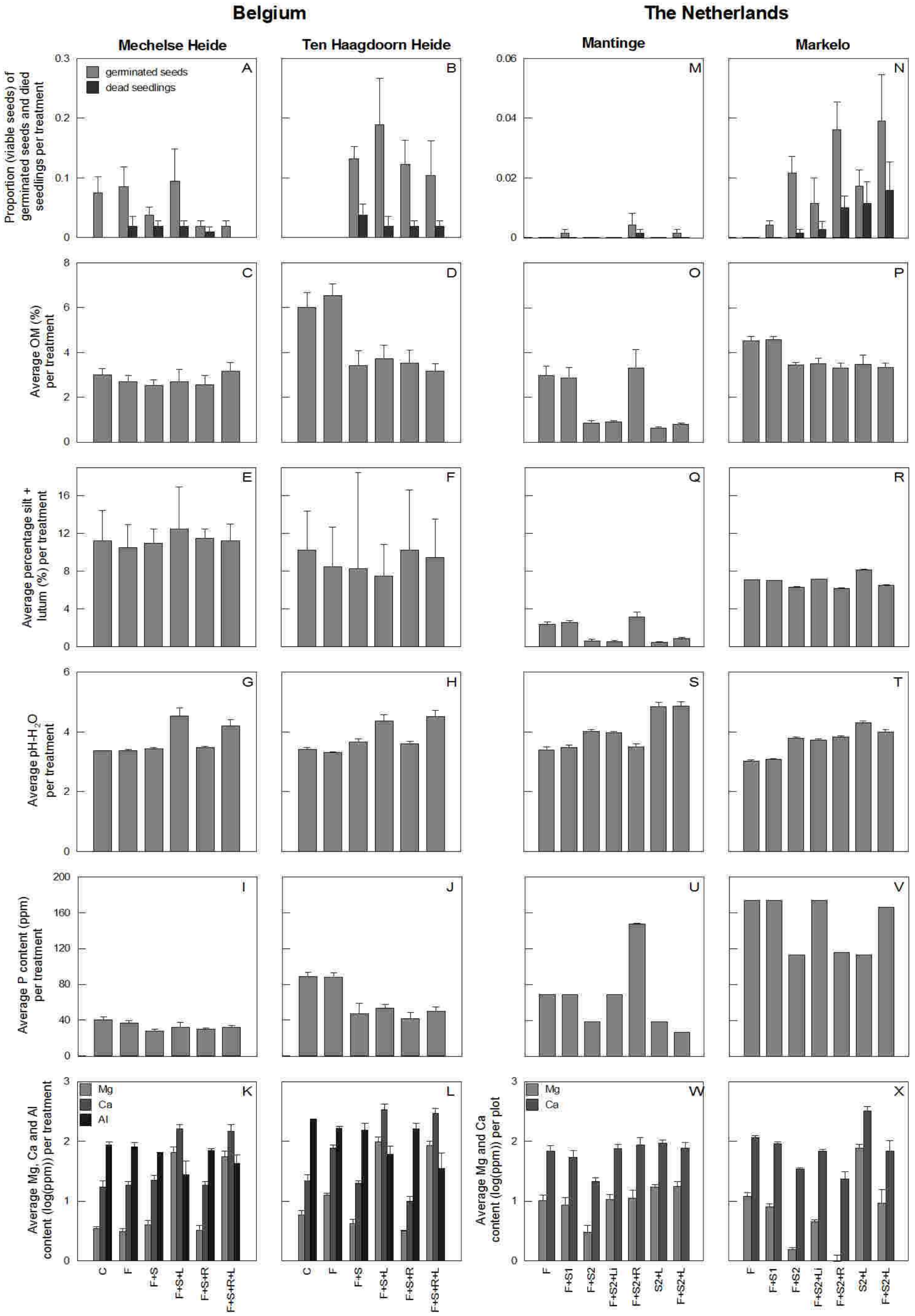

Fig. 7-1. The average proportion of germinated seeds and dead seedlings per treatment and per site and for the different soil variables. Error bars indicate variability. The proportion germinated seeds and died seedlings was calculated by dividing the number of seedlings and died seedling by the number of viable seeds (note the different Y-axis scales in (A), (B), (M) and (N)). (F: fencing; S: deep sod cutting in Belgium; S1: shallow sod cutting; S2: deep sod cutting in the Netherlands; L: liming; R: rotavation; Li: addition of common juniper litter; OM: Soil Organic Matter).

7.3.4 Germination and mortality vs. treatment

In three of the four sites, no seeds germinated where no sod cutting took place (Fig. 7-1 A, B, M, N). A second remarkable fact is the extreme low germination rates in Mantinge, which is likely due to the low loam percentage in that area (ca. 1%) in comparison with the other sites (7% – 15.7%).

Table 7-4. Germination and mortality rates in the different experimental treatments, compared to the control plots. Because no seeds germinated in the control treatments in the Netherlands, F+S1 was used as control comparison for mortality.

Country	Treatment	Germination		Mortality	
		t-value	p-value	t-value	p-value
Belgium	F	0.329	n.s.	0.722	n.s.
	F+S	1.881	n.s.	1.173	n.s.
	F+S+L	2.450	< 0.05	1.024	n.s.
	F+S+R	1.270	n.s.	0.951	n.s.
	F+S+R+L	0.746	n.s.	1.040	n.s.
The Netherlands	F+S1	1.362	n.s.	-	-
	F+S2	2.527	< 0.05	0.389	n.s.
	F+S2+Li	1.242	n.s.	0.772	n.s.
	F+S2+R	3.865	< 0.001	1.724	n.s.
	S2+L	2.247	< 0.05	2.118	< 0.05
	F+S2+L	3.587	< 0.001	1.045	n.s.

n.s.: not significant, F: fencing; S: deep sod cutting in Belgium; S1: shallow sod cutting; S2: deep sod cutting in the Netherlands; L: liming; R: rotavation; Li: addition of litter.

In Belgium, treatment fencing + sod cutting + liming (F+S+L) was most successful, and it was the only treatment that resulted in significantly more seedlings than the control (Table 7-4). However, the significance level is rather low. This trend is less visible in Fig. 7-1. However, this is partly caused by the fact that the data in the figure were split up for the two sites. In the Dutch experiment, no seeds germinated in the control (fenced) plots (F) and two treatments (F+ S2+R and F+S2+L) were most successful (Table 7-4). Two other treatments (F+S2 and S2+L) were also significant better than the control treatment (Table 7-4). Despite the differences in significance in the Dutch experiment, comparisons between these four treatments showed no differences in success (results not shown).

Mortality occurred in all treatments except for F+S1 in the Netherlands (Fig. 7-1 M, N). However, only the non-fenced plots in the Dutch experiment had significant higher mortality rates than the control plots (Table 7-4). In Fig. 7-1 A, B, M and N, this effect is less visible, but when comparing the length of the black bars with the length of the grey bars, it is clear that only for this treatment, more than half of the seedlings died.

7.4 Discussion

The main goal of our research was to assess the effects of heathland management on germination and seedling establishment of common juniper. Second, we aimed to explain recruitment patterns by changing soil conditions under the different management practices. Deep sod cutting in combination with liming or rotavation were most successful to improve recruitment in common juniper. This effect can mainly be explained by the creation of bare soil. Effects of pH and the amelioration of the base saturation degree however, seemed to be more induced by the different soil conditions between the sites than by liming. In addition, low silt + lutum or OM concentrations (which possibly can be linked with drought stress) were revealed as an important factor for germination. We first discuss the differences in seed viability and germination success between the origins of the seeds and between the experimental sites. Second, we explain the different germination rates depending on the treatments and the soil conditions. Finally, the present and coming challenges for common juniper and several practical advises concerning the creation of suitable conditions for juniper recruitment in heathlands are summarized.

Seed viability of common juniper in Belgium and the Netherlands is extremely low. It is unsurprising that seeds that originated from populations with relatively high percentages of viable seeds (e.g. Rossdach) had the best absolute germination success. This is in accordance with the findings of Verheyen *et al.* (2009) who revealed a triangular relationship between seed viability and the percentage recruitment in a population. However, germination rates were still very low (max 1.75 %), regardless of the origin and percentage of viable seeds, which suggests additional causes for failing germination. In the Dutch experiment, germination rates were exceptionally low (0.03 %). The sites and soil treatments were more or less comparable between the two experiments, thus probably other variables such as drought stress and predation were responsible for these differences. Due to these low germinantion rates, the results should be interpreted with caution. However, there are still some trends visible that can be useful for management.

Our results corroborate previous conclusions (e.g. Ward 1973, Clifton *et al.* 1997, Hommel *et al.* 2009) on the need for bare soil for germination of common juniper seeds. Little or no seeds germinated on the plots without sod cutting, except in the Mechelse Heide in Belgium, where the organic layer was rather thin. This result is further corroborated by the plots where litter of common juniper was scattered: only on places where the litter was already decomposed, seedlings could establish.

Secondly, an extreme low silt + lutum portion in the soil and low soil organic matter content seems to have a negative impact on the germination: especially the remaining organic matter in the soil after deep sod cutting must be sufficiently high. This is highlighted by the germination rates and silt + lutum and organic matter contents of the soil in the Mantinge vs. other sites (Fig. 7-1). Moreover, the most successful treatment that included sod cutting in Mantinge, led to relative high soil organic matter and silt + lutum contents (Fig. 7-1 M, O, Q). These findings make it plausible that drought stress is the second most important factor in the failing germination of common juniper seeds, as both soil organic matter (Stevenson & Cole 1999) and silt + lutum (Fitter & Hay 2002) content are responsible for elevated water availability in the soil. In addition, this can also lead to a cooler microclimate. Average silt + lutum concentrations were at least 2.6 % higher in the Belgian sites than in the most successful Dutch site (Markelo). As the nutrient concentrations and pH of the soils were comparable (Fig. 7-1 G, H, T and K, L, X), it is likely that the large differences in germination rates can possibly be explained by lower water availability or a warmer microclimate (in Markelo) and, to a lesser extent, in Mantinge, caused by a lower silt + lutum and/or soil organic matter content.

Although the bare soil and silt + lutum and OM concentrations seem to be two key factors in the germination process, there is still variation in germination success between comparable plots related to these factors. For example, although sod cutting is effective for the creation of bare ground and the removal of excessive nutrients and competing vegetation (e.g. Niemeyer *et al.* 2007), this is not always sufficient to result in significantly higher germination rates (e.g. the Belgian experiment). A combination with management that increases the soil pH seems often necessary. The on-going soil acidification of heathlands, a natural process on sandy soils that is accelerated due to the litter of heath vegetation and due to atmospheric deposition of mainly nitrogen and, today to a lesser extent, sulphur (Roelofs 1986, Aerts *et al.* 1991, Uren *et al.* 1997), is associated with a decrease of exchangeable base cations and the increase of exchangeable soil Al^{3+} concentrations (Bownman *et al.* 2008). Also in our study area, pH-KCl-values of soils in the control plots were lower than 3.6 (i.e. a pH-H_2O of 4.4 after conversion sensu Azedevo *et al.* (2013)) and situated within the aluminium and iron buffer range (pH-H_2O < 4.5; Bowman *et al.* 2008). Thus, there is a risk of negative toxic effects of Al^{3+}, for example on plant roots, with typical symptoms as root length reduction, a dying off of the root tip meristem and a reduction of the Ca^{2+}- and Mg^{2+}-uptake (Runge & Rode 1991). Sod cutting up to the mineral soil can increase the pH due to the removal of the acidic top layer (Van Den Berg *et al.* 2003). This was most pronounced in the deep sod cut treatment in the Netherlands. However, pH values remained rather low, and, in Belgium, when not limed, pH values hardly reached the cation exchange buffer range (max soil pH-H_2O of 4.7). In addition, at low pH values, sod cutting can have a negative impact on germination as the removal of organic compounds reduces the capacity of complexing the toxic aluminium (Van den Berg *et al.* 2003) and acidification accelerates.

Former studies therefore proposed additional liming after sod cutting to increase soil pH and base saturation (De Graaf *et al.* 1998, Van den Berg *et al.* 2003). In our study, liming had a strong positive effect on the pH. In Belgium, liming was necessary to lead to significantly better germination rates than in the control plots. However, in the Netherlands, there was no difference in germination success between the two other successful treatments without liming (F+S2 and F+S2+R; results not shown). It is possible that the subtle higher pH after deep sod cutting in the Netherlands compared to the Belgian experiment can explain this effect. This means that in the Belgian experiment, extra liming is needed to sufficiently raise the pH. Surprisingly, rotavation in Belgium seemed to neutralize the influence of liming. Increase of pH and cation-concentrations were accompanied by a strong decrease of Al^{3+}-concentrations in Belgium. These findings support the assumption of Al^{3+} toxicity inhibiting germination and seedling establishment of common juniper. The treatment with both deep sod cutting and rotavation in the Netherlands (F+S2+R) led to a strong pH increase. Probably this effect was generated by the combination of the removal of the acid top layer and the mixture with less acidic subsoil. A positive consequence is that the soil organic matter and silt + lutum content did not significantly decrease, probably due to mixture with organic matter from the deeper soil. Both soil organic matter and silt + lutum are important as they improve the buffering capacity of the soil. In other successful treatments, silt + lutum and/or soil organic matter content are lowered. Thus, it is possible that the positive effects of liming and deep sod cutting are not sustainable due to a lower capacity of the soil to retain a large amount of cations. In addition, if too much liming causes a high increase of the soil pH, it can reduce the soluble phosphorus by forming insoluble Ca phosphates (White & Taylor 1977, Stevenson & Cole 1999). This in turn can negatively affect the seedling survival. Liming had no significant effects on seedling survival. However, the mortality could be relatively high in limed plots, especially in the Netherlands (Fig. 7-1). Therefore, this treatment should not be applied ill-considered. Although rotavation seems a good option to create suitable germination conditions, this management action should be done with caution as the roots of adult, extant common juniper shrubs can be damaged when they are approached too near (Verheyen *et al.* 2005).

The treatments had no significant effect on the survival of the seedlings except for the plots outside the fence in the Dutch experiment. In the Belgian experiment, the plots outside the fence were covered with other vegetetation (no sod cutting). Hence, the germinated seeds were less visible to herbivores such as deer or rabbits. In the Netherlands, seedlings outside the fence had no cover and, thus, were an easy meal. Grazing sheep, cattle, deer or rabbits can lower seedling survival and successful recruitment of common juniper (Ward 1973, Clifton *et al.* 1997). For example, collapses of rabbit populations by myxomatosis marked periods of expansion of many common juniper populations in the UK (Ward 1973). Thus, fencing can support the survival of juniper seedlings, especially when they germinate after a sod cutting treatment.

7.4.1 Conclusions

Our results reveal a complex relationship between recruitment success, soil conditions and management. The most important prerequisite seems to be that seeds have contact with the mineral soil to allow germination. A second important factor is probably water availability or a cool microclimate. Soils with low percentages in organic matter and/or silt + lutum are less suitable for germination. Actions that lower those percentages, as deep sod cutting, should be avoided at drought-stressed locations. When both restrictions are met, the soil pH of the top soil is an important characteristic to determine whether liming is a possibility or not, although this measure should be taken with caution on nutrient poor soils.

Our study highlights the precarious condition of common juniper in the northwestern European lowlands. In this region, the species suffers from an extremely low seed viability, which can be explained by the negative influence of increasing temperatures and nitrogen deposition (Verheyen *et al.* 2009, Chapter 4). These negative effects on seed viability are enhanced by the eutrophying and acidifying effects of atmospheric deposition on heathlands (Krupa 2003), deteriorating germination conditions. However, despite low seed viability, seedlings of local origins were found in our experiments. Therefore it is still useful to create optimal germination conditions on places where higher seed input can be expected: near the mother shrub (although conditions there are less favourable due to direct competition) and under or near possible roosting places for birds that tend to disperse juniper seeds (mainly trushes (*Turdus* spp.); Bergman 1963, Livingston 1972, Breek 1978, García 2001) such as large stones, solitary trees or forest edges (Livingston 1972, García 2001, Hommel 2009). Additional fencing will probably improve survival chances for the seedlings. Nevertheless, a significant higher input of viable seeds is necessary to lead to sustainable populations: with the spectacularly limited number of seedlings in our experiment (176 seedlings emerged from *ca*. 260,491 seeds sown), one average mother tree would hardly produce one seedling per year.

8 General discussion and conclusion

The last decades, the size and number of common juniper populations are declining in several parts of Europe. Lack of recruitment due to a failing sexual reproduction is an important reason for this decline.

Here we assessed the effects of several global-change drivers on crucial phases of the sexual reproductive phase using both observational and experimental approaches. First, we determined which of three seed developmental phases were most crucial (Chapter 3). Then we studied how different global-change drivers were correlated with seed viability during the two important phases (Chapters 4-6). Finally, in Chapter 7, since low seed viability is not the only reason for the lack of recruitment, we determined the optimal soil conditions for seed germination and seedling survival. In this closing chapter, we first briefly summarise our findings and discuss management implications, using Flanders (northern Belgium) as a case study. We end with some recommendations for further research.

8.1 Sexual reproduction in common juniper

8.1.1 Crucial seed phases

We focussed on three crucial seed phases during the sexual reproductive cycle of common juniper: (seed phase 1, SP1) the early gametophyte development and pollination; (seed phase 2, SP2) further development of the gametophytes, fertilization and early embryo development; (seed phase 3, SP3) late embryo development. In general, we found a progressive and drastic reduction of seed viability over the three seed phases (on average, 59, 23 and 9 % viable seeds after SP1, SP2 and SP3 respectively). There are multiple reasons possible for this decline: the lack of pollination (Owens 1995) and frost damage (Owens 2006) (SP1), disturbed growth of the pollen tube and/or female gametophyte (Owens *et al.* 2001, Fernando *et al.* 2005) which, in addition, can result in a disturbed male-female synchrony within the developing seed (Herrero 2003) (SP2), disturbed development of the megagametophyte and embryo (SP3). Although we found anomalies after SP1 (i.e. lack of pollination or a shrivelled ovule), they could not explain the presence or absence of recruitment in the different populations. We also didn't find indications that pollen viability could be a problem (Box 1). Therefore, this seed phase was not further studied. Only after SP3, we found significant differences in seed viability between populations with and populations without recruitment (Chapter 3). Hence, this made us conclude that late embryo development is the most crucial phase during seed development. However, anomalies during SP2 can have a delayed effect on embryo development (Owens 1995, Owens & Morris 1998). Therefore, both seed phases were retained for further research. In addition, a complex relationship between the ripening time of the seeds of common juniper and the seed viability appears to exist. Most seeds ripening in three years already have low viability shortly after fertilization, whereas the viability of seeds ripening in two years decreased mostly during embryo development. Shorter ripening times allow for less time for external environmental factors to interact with the seed and negatively affect seed viability. This can explain why seed viability of SP2 seeds that ripen in less than three years is relatively high. However, due to a faster development during SP2 some processes are possibly not completed or the male-female synchrony is disturbed, leading to anomalies during SP3. This can explain the strong decrease in viability after SP3 for seeds that ripen in less than three years. Thus, in our further research it was also important to zoom into the effects of global-change drivers on ripening time on the one hand and the effect of ripening time on seed viability on the other hand.

8.1.2 The effect of increasing temperatures and enhanced atmospheric depositions on the viability of common juniper seeds

Both increasing temperature and enhanced atmospheric depositions were negatively correlated with seed viability of juniper (Chapters 4 – 5). In addition, in Chapter 6 we experimentally confirmed the negative effects of increasing temperatures on seed viability.

There are different pathways through which these global-change drivers possibly elaborate these negative correlations. For example, increasing temperatures can negatively affect the development of the female gamethophyte or other female structures (e.g. Kozai *et al.* 2004, Hedhly *et al.* 2003), the growth of the pollen tube (through its effects on the female structures; e.g. Fernando *et al.* 2005), the male-female synchrony in the pre-fertilization phases (Zinn *et al.* 2010, Hedhly 2011), the development of the megagametophyte and the nutrition and growth of the embryo. On the other hand, both atmospheric depositions and increasing temperatures can cause nutrient imbalances (Chapter 5). For example, faster growth which is induced by higher temperatures can generate a dilution effect on the internal N, P and S pool (e.g. Doiron *et al.* 2014). Enhanced atmospheric depositions possibly lead to a decreased uptake and leaching of cations (e.g. Krupa 2003). Also the reduction of mycorrhizal associations caused by atmospheric deposition (Malcová *et al.* 1999; Krupa

2003) can help to explain the lower P-concentrations in the needles of common juniper. In addition, we found that a shortage of P, Mg and Ca, key elements during the nutrient storage throughout the seed development (Marschner 1995), can lead to anomalies and seed abortion.

Although the ripening of the embryo (SP3) was identified as the most vulnerable phase, we found strong indications that the actual reasons should be searched in the preceding processes (e.g. the development of the male and female gametophyte, fertilization and early embryo development, i.e. SP2). For example, the transplant experiment (Chapter 6) clearly showed that significant differences in seed viability between the temperature treatments only occurred if the seeds also endured higher temperatures during SP2. Hence, it is plausible that anomalies, induced by increased temperatures or enhanced atmospheric deposition, occur during the development of the female gametophyte (SP2). However, their effects only manifested themselves in SP3, for example by leading to a malfunctioning megagametophyte that will not be able to nourish the developing embryo.

Enhanced atmospheric depositions and warming were related with shorter seed ripening times. This phenomenon is hard to explain, as, to our knowledge there are no other plants where seeds can ripen in two or in three years, and where both types of ripening strategies can be found within one individual. Being a cold adapted species (*cf.* its relatively northerly distribution range and the assumption of its survival in Central Europe during the last glacial maximum; Michalczyk *et al.*, 2010), there is a probability that the mechanism of delayed fertilization to overcome shorter reproduction seasons has developed in common juniper (Willson & Burley, 1983). Longer reproduction seasons due to higher temperatures can reduce the need for such mechanisms, leading to a higher chance of shorter ripening times. Nevertheless, using the large scale sampling data of Chapter 4, we found no important effect of ripening time on the seed viability.

8.1.3 Effects of management actions on the recruitment

Although low seed viability is a major restriction in the sexual reproduction of common juniper, Verheyen *et al.* (2009) revealed that low recruitment can also have other causes. Hence, if seed viability is high, e.g. a suitable microclimate for establishment or the absence of grazing is still important. In Chapter 7, we showed that soil conditions, influenced by management actions, played an important role in seed germination and seedling survival. Most importantly, we found that the seeds needed to be in contact with mineral soil to be able to germinate (see also e.g. Clifton *et al.* 1997). For example, sod cutting can create these conditions. A second important factor is probably water availability or a cold microclimate. Soils with low percentages in organic matter and/or silt + lutum have a lower capacity to retain water (Stevenson & Cole 1999, Fitter & Hay 2002) and are less suitable for germination. In sites with low silt + lutum concentrations, actions that lower the organic matter in the soil, such as deep sod cutting, should be avoided to prevent drought-stress. When both restrictions are overcome, the soil pH of the top soil is an important characteristic to determine whether liming is a possibility or not. Nonetheless, this measure should be taken with caution on nutrient poor soils as too much liming can cause a high increase of the soil pH, which can reduce the soluble phosphorus by forming insoluble Ca phosphates (White & Taylor 1977, Stevenson & Cole 1999). This, in turn, can have a negative impact on the seedling survival.

8.2 Management implications

We will discuss the management implications of our findings using a case study of juniper in Flanders (see Gruwez *et al.* 2010b). In 2008, we assessed the conservation status of 68 of the 72 remaining common juniper populations in Flanders. Via a simple, deterministic model (i.e. no randomness is included), we calculated how different management actions could have an influence on this conservation status after a period of 23 years (Fig. 8-1).

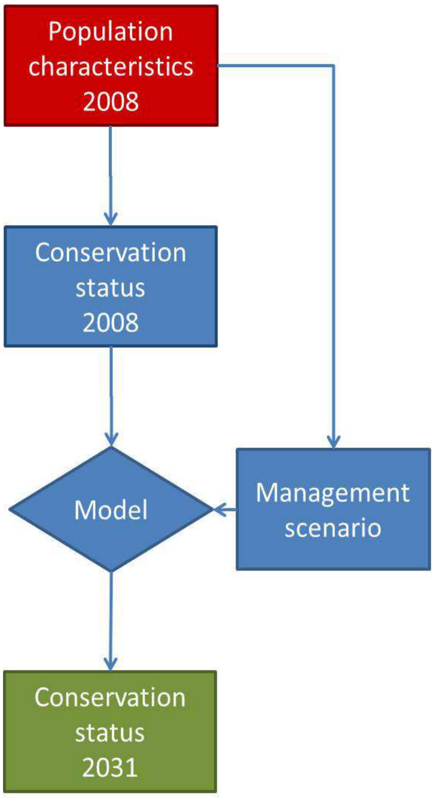

Fig. 8-1. Schematic overview of the management model. Based on the population characteristics in 2008, the conservation status of the common juniper populations in 2008 is assessed. Population characteristics also determine which management actions will be applied in the different populations. The model calculates how the different management actions change the conservation status of the populations after 23 years.

In brief, in this model we calculated the number of individuals in each population after 23 years with the following formula:

$$A = X*(1-m) + Y*r$$

With A being the number of individuals after 23 years, X the number of individuals in 2008, Y the number of female individuals in 2008, m the mortality rate and r the proportion of recruiting individuals produced by a female shrub in 23 years. Both m and r can be influenced by different management actions and environmental variation (more information is available in Gruwez *et al.* 2010b).

The assessment of the conservation status was based on six population characteristics: (1) number of individuals per population, (2) percentage viable shrubs, (3) percentage female shrubs, (4) recruitment (number of shrubs smaller than 0.5 m), (5) percentage soil cover and (6) canopy closure above the juniper shrubs (Table 8-1). The number of individuals in a population is an important measure for its survival. A population needs a minimum of individuals to maintain itself (Shaffer 1981). Ward (1973) considered 100 individuals as a minimum for a viable common juniper population. In addition, the viability of the shrubs themselves is also important for the survival of the total population. Furthermore, the input of seeds (via female shrubs) and the presence of recruitment to replace at least the parent shrubs are essential for a sustainable population. Finally, soil cover and canopy closure have an influence on the establishment of seedlings and the viability of the shrubs, respectively.

Table 8-1. Overview of the features and thresholds used to determine the conservation status. (A) good, (B) sufficient, (C+) moderately degraded, (C–) highly degraded, (┼) extinct.

	Conservation status				
Characteristic	A	B	C+	C-	┼
Number of individuals*	≥ 100 ex.	≥ 100 ex.	$5 \leq n < 100$	$1 \leq n < 5$	0
Percentage vital shrubs(1)	≥ 75 %	$50 \leq x < 75\%$	$25 \leq x < 50$ %	$< 25\%$	
Percentage female shrubs	$20 \leq x \leq 80\%$	$20 \leq x \leq 80\%$	$> 80, < 20\%$	$> 80, < 20\%$	
Recruitment(2)	More juveniles than adults	Less juveniles than adults	No recruitment	No recruitment	
Percentage soil cover	$< 25\%$	$25 \leq x < 50\%$	$50 \leq x < 75\%$	$\geq 75\%$	
Percentage canopy closure	$< 25\%$	$25 \leq x < 50\%$	$50 \leq x < 75\%$	$\geq 75\%$	

(1) vital shrub = shrub with less than 10% needle loss; (2) recruitment = number of juveniles (young shrubs smaller than 0.5 m); * adult individuals

The characteristic with the lowest value determines the overall status of the population. To estimate the future status of a population, it is necessary to determine the expected recruitment and the mortality. By using our own data and data from Van Dijk (1982), we calculated that, under optimal germination conditions, one female shrub annually produces 0.006 vital, reproducing individuals, assuming a seed viability of 2.5 % (based on the seed viability in the Flemish populations in Chapter 3 and 7), a germination percentage of 8.3 % (Chapter 7), a survival after ten years of 0.006 (Van Dijk 1982) and an average of 4774 seeds per shrub (based on the results of the Flemisch populations in Chapter 3). To incorporate the effects of climate change in our model, we also calculated an alternative factor taking in to account a decrease in seed viability of 96 % when temperatures rises about 2.6 °C (based on the results for seed viability in Belgium low elevation and Southern France in Chapter 6). In that case, each female shrub annualy produces only 0.0002 vital, reproducing individuals.

Mortality was estimated by using data from Verheyen *et al.* (2005) who found that in a period of 23 years, 46 % of the shrubs in Heiderbos in As (Flanders), had died.

We then assessed the influence of three management actions on the future status of the populations: (i) decreasing crown closure if crown closure was higher than 25%, (ii) creating bare soil in populations with a percent ground cover of vegetation higher than 25 %, and (iii) plant additional young shrubs until the population reaches a size of 100 individuals. In our model, opening op the crown cover to less than 25 %, decreased the chance of mortality with 10 %. Creating 25 % bare soil in the population made recruitment possible (Chapter 7). Hence, in populations where the area of open soil was less than 25 % of the total population area, female shrubs produced annually zero individuals. The third management action (increasing the population size), called ‘translocation’, is often applied and necessary to create sustainable populations of juniper (Gordon 1996, Menges 2008). For northwestern Europe, in theory, the restrictions for the origin of the plant material are rather low as all the common juniper populations in this region are virtually indistinguishable in terms of their gene pool (Vanden Broeck *et al.* 2010). If local adaptations are absent for common juniper, one could consider to use plant material in transplantations originating from the south to tackle problems such as climate change (‘assisted migration’). Although we found no indications for local adaptation in common juniper (see Chapter 6), further research is needed to give a conclusive answer on this topic. Hence, for the moment, it remains recommended to use plant material of local origin to conserve genetic diversity.

We studied the effects of three management scenarios on the populations in Flanders: (1.1) a business-as-usual scenario, where none of the management actions is applied; (1.2) a scenario where necessary crown closure is decreased and bare soil is created; and (1.3) where necessary, crown closure is decreased, bare soil is created and the number of shrubs is increased up to 100 individuals. The same scenarios were repeated using the correction for climate change (scenario 2.1, 2.2 and 2.3).

For the scenarios without climate change, this modelling exercise highlighted only minor differences between a business-as-usual scenario (scenario 1.1) and the scenario where only canopy closure and open soil are treated (scenario 1.2) (Table 8-2). In both scenarios, about a quarter of the Flemish populations will disappear by 2031. However, in scenario 1.2, a higher amount of the populations gets a C+ quotation.

Table 8-2. The number of populations per type of conservation status (a) and per limiting factor for a good conservation status (i.e. the factors that have a status below A) (b), in 2008 and for the different management scenario’s in 2031. Scenarios 1.1, 1.2 and 1.3 do not take climate change into account, scenarios 2.1, 2.2 and 2.3 take a temperature increase of 2.6 °C into account. (A) good, (B) sufficient, (C+) moderately degraded, (C–) highly degraded, (┼) extinct.

	2008	2031 (different scenarios)					
		1.1	1.2	1.3	2.1	2.2	2.3
(a) Conservation status							
A	0	0	0	0	0	0	0
B	0	0	0	47	0	0	3
C+	8	6	19	18	6	19	62
C-	60	44	32	3	44	32	3
┼	0	18	17	0	18	17	0
Number of surviving populations in Flanders	68	50	51	68	50	51	68
(b) Limiting factor for a good conservation status (several factors possible per population)							
Number of individuals	62	65	65	18	65	65	62
Percentage vital shrubs	58	55	55	4	55	55	4
Percentage female shrubs	44	50	46	4	50	47	4
Recruitment	67	68	68	68	68	68	68
Soil cover	45	45	0	0	45	0	0
Crown closure	47	47	0	0	47	0	0

In contrast, scenario 3 (canopy closure, bare soil and planting extra individuals) saves all populations from extinction (which is not surprising) and increases the conservation status of most of the populations to a B-level. However, none of the populations reaches a good conservation status (A). The most important reason for this failure is the low recruitment in all the populations (Table 8-2b). Hence, management focused on common juniper conservation e.g. creating good growth conditions and an ideal microclimate for germination and seedling survival is undoubtable necessary and useful to maintain sustainable juniper populations. However, in regions where recruitment is absent due to very low seed viability, enforcing the populations or creating new populations by planting seems inevitable.

If we take temperature increase of 2.6 °C into account, the results for scenarios 2.1 and 2.2 are the same as for scenarios 1.1 and 1.2. The lack of recruitment was already an important reason for the low status of the populations and this remains the same. However, in scenario 2.3, only three populations reach a conservation status of B and in 62 populations, the low number of individuals is one of the main reasons for this quotation (compared to 18 in scenario 1.3). Seed viability is so low in a climate change scenario that, already after 23 years, the discrepancy between recruitment and mortality lowers the number of individuals in a population below 100. Hence, due to climate change, efforts to ameliorate the conservation status of common juniper populations will have to increase even more.

To conclude this section, we propose a few guidelines that can help to preserve common juniper in the future in Belgium specifically and similar regions in northwestern Europe:

- Overall, this work is an extra argument to policy makers to increase their efforts in reducing the emissions of green house gases and nitrogen (e.g. in traffic, industry and agriculture).
- Focus the efforts for creating sustainable populations on large areas where the management is already suited for common juniper (e.g. heath and calcareous grasslands);
- Seed viability is negatively correlated with temperature and atmospheric depositions. Hence, it can be interesting to pay special attention to locations where the microclimate is expected to be colder (e.g. northerly oriented slopes or the vicinity of small rivers) and where depositions are lower (e.g. not to close to highroads and farms);
- To stimulate establishment of new individuals from natural recruitment, it is necessary to create gaps in the vegetation with bare soil. In addition, to avoid drought stress, the amount of organic matter or silt and lutum should not be too low (>3 % and > 8 % respectively). Ideally, the pH should reach the cation exchange buffer range (about 4.7). Hence, additional liming can be an option. However this measure should be taken with caution on nutrient poor soils;
- Younger individuals seem to have a higher percentage of viable seeds (see e.g. Chapter 4 and 6). Hence, it is useful to identify locations with seedlings and protect them from grazing by e.g. rabbits or deer;
- Finally, successful recruitment in common juniper will only occur if several of the following conditions are met: enough viable seed must be available (e.g. during a colder period of several years), enough microsites suitable for establishment must be present (e.g. by sod cutting, but also by other disturbances) and there should be enough dispersers (e.g. a short time invasion of *Turdus pilaris*). Hence, the larger the area with suitable habitat, the higher the chances that locations exists where all these conditions occur at the same time. In this respect, it is possible that due to habitat loss

in the past, chances for recruitment started to decrease. This could have led to an increase of the average age of the shrubs, which in turn can have resulted in a lower seed viability (see Chapter 4 and 6). Although this process is hypothetical, it still stresses the importance of protecting young juniper shrubs.

8.3 Perspectives for further research

Based on the different findings in this thesis, a number of suggestions to further our understanding of the low seed viability and problematic recruitment of common juniper are proposed.

First, to further unravel the mechanisms behind the negative effects of increasing temperatures and enhanced atmospheric depositions additional research is needed at the bio(geo)chemical level. It can be useful to study the effect of these global change drivers on the biochemical composition (e.g. amino acids, starch, lipids, …) of the female gametophyte (and in later phases the megagametophyte) and the embryo. A second step in this research would be to link this biochemical composition to seed viability. An experiment with young clones of common juniper that are experimentally grown at contrasting levels of temperature and atmospheric depositions could answer these questions. Seeds should then be sampled regularly. In a final stage, the percentage viable, ripe seeds should be assessed and linked to both the treatments and the biochemical composition of the seeds that were sampled in earlier phases.

Secondly, in our transplant experiment (Chapter 6), we found that the seed viability on the shrubs was high, compared to seeds that were sampled in natural populations. The reason for this surprising pattern was not clear, but there are some possible questions that can be addressed. For instance, studies including young shrubs vs. old shrubs, cuttings vs. natural regeneration from seeds, rich soil vs. poor soil could be performed. Experiments in which different fertiliser treatments are applied to adult shrubs, cuttings and natural regeneration from seeds can help to explain the differences in seed viability.

Finally, it is often mentioned that grazing by e.g. sheep and cattle can help to achieve ideal conditions for the establishment of common juniper (e.g. Fitter & Jennings 1975, Hommel 2009). In addition, the presence of common juniper shrubs is often associated with sheep walks (Ward 1973, Rosén 1988). However, this management also risks damaging young and mature juniper shrubs (Ward 1973, Clifton *et al.* 1997). The question remains if grazing is a good management option to promote recruitment of common juniper. A large scale sowing experiment in grazed and ungrazed plots could answer this question. Our findings in Chapter 7 emphasize that it is important to use seeds that are sampled in populations with a high seed viability. If seed viability is low, chances for germination are also low and then, there is a risk that possible trends cannot be supported by statistics.

Appendix A

Table A. Correlations between seed quality, cone age, cone phase and seed phase for the different populations

Population			Cone age	Cone phase	Seed phase
Ekulunde	Seed quality	Correlation Coefficient	-.233(**)	-.168(**)	-.202(**)
		N	406	406	406
	Cone age	Correlation Coefficient		.586(**)	.525(**)
		N		464	406
	Cone phase	Correlation Coefficient			.801(**)
		N			406
Kleszczele	Seed quality	Correlation Coefficient	-.338(**)	-.400(**)	-.375(**)
		N	403	403	403
	Cone age	Correlation Coefficient		.745(**)	.739(**)
		N		421	404
	Cone phase	Correlation Coefficient			.889(**)
		N			404
Rossdach	Seed quality	Correlation Coefficient	-.215(**)	-.138(**)	-.202(**)
		N	477	477	477
	Cone age	Correlation Coefficient		.657(**)	.717(**)
		N		486	477
	Cone phase	Correlation Coefficient			.931(**)
		N			477
Asholdinger	Seed quality	Correlation Coefficient	-.140(*)	-.247(**)	-.284(**)
		N	312	312	312
	Cone age	Correlation Coefficient		.646(**)	.784(**)
		N		322	312
	Cone phase	Correlation Coefficient			.890(**)
		N			312
Zutendaal	Seed quality	Correlation Coefficient	-.410(**)	-.328(**)	-.449(**)
		N	390	390	390
	Cone age	Correlation Coefficient		.431(**)	.573(**)
		N		402	390
	Cone phase	Correlation Coefficient			.857(**)
		N			390
As	Seed quality	Correlation Coefficient	-.366(**)	-.484(**)	-.471(**)
		N	452	452	452
	Cone age	Correlation Coefficient		.765(**)	.784(**)
		N		456	452
	Cone phase	Correlation Coefficient			.988(**)
		N			452
Markelo	Seed quality	Correlation Coefficient	-.286(**)	-.486(**)	-.460(**)
		N	439	439	439
	Cone age	Correlation Coefficient		.701(**)	.666(**)
		N		465	440
	Cone phase	Correlation Coefficient			.953(**)
		N			440
Mantinge	Seed quality	Correlation Coefficient	-.294(**)	-.450(**)	-.489(**)
		N	459	459	459
	Cone age	Correlation Coefficient		.662(**)	.651(**)
		N		488	461
	Cone phase	Correlation Coefficient			.948(**)
		N			461

* $p < 0.05$; ** $p < 0.001$

Appendix B1

Legend of the Table B1 (see next page)

(*) No data available

(1) Limnological Station, Borucino - Meteorology and Climatology Department - University of Gdansk, Poland

(2) Klein Tank, A.M.G. and Coauthors, 2002. Daily dataset of 20th-century surface air temperature and precipitation series for the European Climate Assessment. Int. J. of Climatol., 22, 1441-1453.

(3) Données du SLF © 2012, SLF

(4) Agenzia Regionale per la Prevenzione e Protezione Ambientale del Veneto, Dipartimento per la Sicurezza del Territorio, Centro Meteorologico - Ufficio Validazione Dati e Climatologia

Table B1. Additional information on the sampled populations (populations are ordered from low to high latitude).

Population number	Year of sampling	Country	Latitude (°)	Longitude (°)	Elevation (m above sea level)	Number of sampled shrubs	Number of sampled SP2 seeds	Number of sampled SP3 seeds	Weather station	Source weather data
1	2010	Spain	41.79	-2.839	1240	5	176	52	Soria	http://eca.knmi.nl/ (2)
2	2010	Spain	43.02	-6.228	1420	5	149	73	Leon Virgen del Camino	http://eca.knmi.nl/ (2)
3	2010	Italy	43.232	11.176	320	5	173	124	Siena Orto Botanico	http://www.biometeo.it
4	2010	France	43.26	0.87	300	5	167	122	Toulouse-Blagnac	http://eca.knmi.nl/ (2)
5	2010	Italy	43.456	11.417	358	5	171	117	Gaiole in Chianti	NA (*)
6	2010	France	43.76	6.369	1285	5	167	97	Comps-sur-Artuby	http://france.meteofrance.com/
7	2010	France	43.815	6.246	692	5	122	84	Aiguines	http://france.meteofrance.com/
8	2010	France	43.979	5.473	1100	5	166	121	Saint Christol	www.meteofrance.com
9	2010	France	44.032	5.349	810	5	191	100	Saint Christol	www.meteofrance.com
10	2010	France	44.101	5.45	1007	5	162	118	Saint Christol	www.meteofrance.com
11	2010	France	44.864	5.479	1500	5	224	9	IIserech2	http://eca.knmi.nl/ (2)
12	2010	Italy	45.871	11.653	619	5	163	80	Pove del Grappa loc. Pra' Gollin	http://www.arpa.veneto.it (4)
13	2010	Italy	45.888	11.474	1095	5	163	99	Asiago (aeroporto)	http://www.arpa.veneto.it (4)
14	2010	Switzerland	46.861	9.229	2128	3	69	63	Crap Masegn - Schneestation	http://www.slf.ch/ (3)
15	2010	Austria	47.272	11.265	805	5	105	159	Innsbruck-Flughafen	ZAMG Tirol und Vorarlberg
16	2008	Germany	47.863	11.508	550	7	126	173	Muenchen	http://eca.knmi.nl/ (2)
17	2010	France	49.786	2.305	100	5	142	123	Glisy	http://france.meteofrance.com/
18	2010	Germany	49.973	11.308	450	5	163	109	Bamberg	http://www.dwd.de/
19	2008	Germany	50.021	11.085	440	8	153	260	Bamberg	http://www.dwd.de/

Population number	Year of sampling	Country	Latitude (°)	Longitude (°)	Elevation (m above sea level)	Number of sampled shrubs	Number of sampled SP2 seeds	Number of sampled SP3 seeds	Weather station	Source weather data
20	2010	Belgium	50.1	5.185	345	5	173	116	Humain	http://www.kmi.be
21	2010	Germany	50.211	6.384	595	5	191	106	Schneifelforsthaus	http://www.dwd.de/
22	2010	Belgium	50.443	5.868	535	5	161	95	Mont-Rigi	http://www.kmi.be
23	2010	Germany	50.755	8.32	419	5	174	122	Dillenburg	http://www.dwd.de/
24	2008	Belgium	50.92	5.554	80	7	155	197	Ell	http://eca.knmi.nl/ (2)
25	2008	Belgium	51.017	5.577	75	8	168	277	Ell	http://eca.knmi.nl/ (2)
26	2010	UK	51.142	-1.643	120	5	162	139	Boscombe down	http://eca.knmi.nl/ (2)
27	2010	UK	51.226	-0.437	175	5	117	171	Mickleham	http://www.metoffice.gov.uk/
28	2010	Netherlands	51.549	6.012	50	5	175	112	Arcen	http://eca.knmi.nl/ (2)
29	2010	Germany	51.948	13.491	115	5	143	140	Baruth	http://www.dwd.de/
30	2008	Netherlands	52.28	6.52	50	8	126	246	Hupsel	http://eca.knmi.nl/ (2)
31	2008	Poland	52.594	23.343	100	7	131	228	Brest	http://eca.knmi.nl/ (2)
32	2008	Netherlands	52.792	6.625	50	8	159	260	Hoogeveen	http://eca.knmi.nl/ (2)
33	2010	Germany	52.929	9.353	40	5	162	130	Rotenburg	http://www.dwd.de/
34	2010	Poland	54.196	17.965	190	6	148	116	Limnological Station, Borucino	http://www.ug.gda.pl (1)
35	2010	Ireland	54.456	-8.374	19	5	171	104	Ballyshannon	http://eca.knmi.nl/ (2)
36	2010	Sweden	55.665	13.371	70	5	147	122	Lund	http://www.smhi.se/
37	2010	Sweden	56.537	16.474	45	5	157	128	Ölandsbron	http://www.smhi.se/
38	2008	Sweden	56.576	16.604	45	8	287	55	Ölandsbron	http://www.smhi.se/
39	2010	Sweden	59.184	18.375	4	5	165	97	Stockholm	http://www.smhi.se/
40	2010	Sweden	59.848	17.778	36	5	126	122	Uppsala	http://www.smhi.se/
41	2010	Norway	62.225	9.548	1030	5	144	105	Fokstugu	http://met.no/
42	2010	Sweden	64.233	19.785	179	5	115	62	Vindeln-Sunnansjönäs	http://www.smhi.se/

Appendix B2

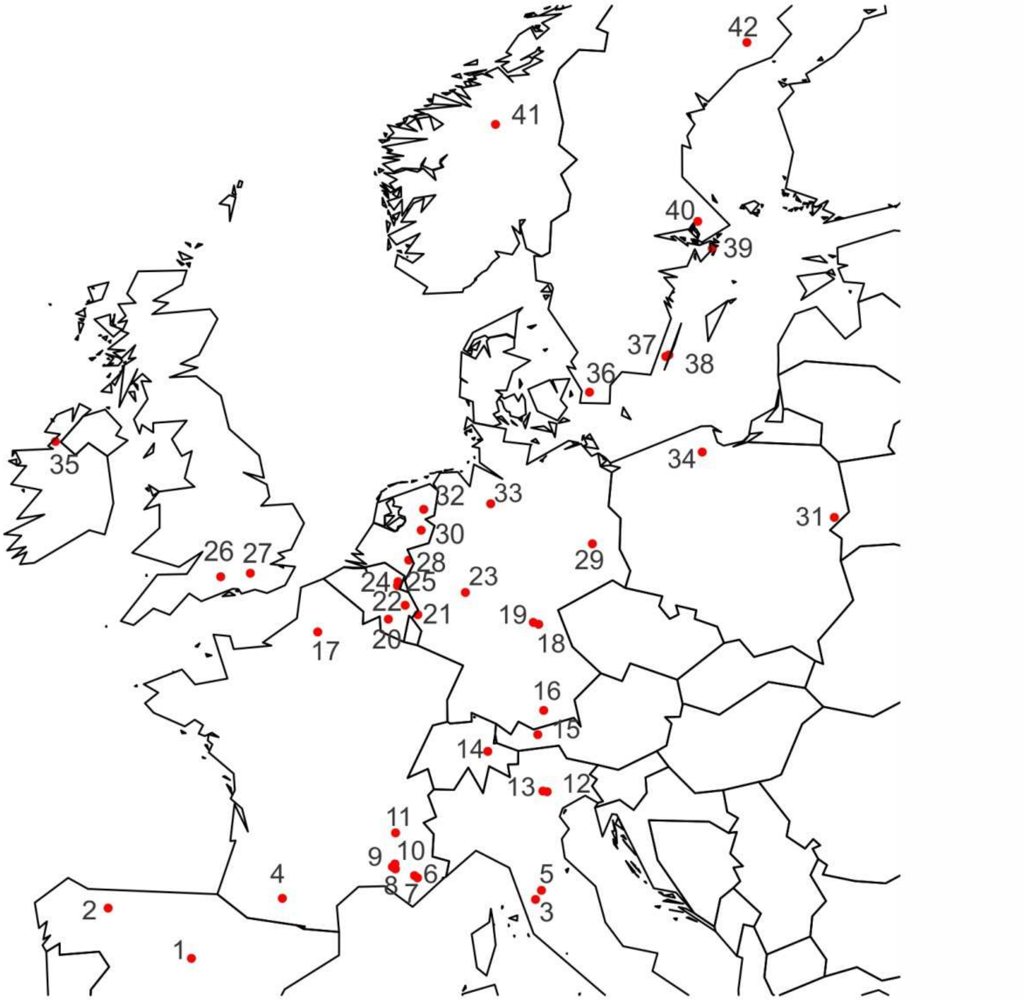

Fig B2. Location and population number of the sampled common juniper populations (for information on the populations, see Appendix B1).

Appendix B3

Table B3. Detail of the seasons that were taken into account for calculation the GDD>0°C of the spring of pollination, the summer of fertilization and the year of embryo development for SP2-seeds with a ripen time of three years (SP2RT3) and two years (SP2RT2) and for SP3-seeds with a ripen time of three years (SP3RT3) and two years (SP3RT2).

	Season (*)	SP2RT3	SP2RT2	SP3RT3	SP3RT2
Year of sampling	Winter				
	Autumn			embryo development	embryo development
	Summer	fertilization	fertilization	embryo development	embryo development
	Spring		pollination	embryo development	embryo development
	Winter			embryo development	embryo development
1 year before sampling	Winter			embryo development	embryo development
	Autumn			embryo development	embryo development
	Summer			fertilization	fertilization
	Spring	pollination			pollination
	Winter				
2 years before sampling	Winter				
	Autumn				
	Summer				
	Spring			pollination	
	Winter				

(*) Winter (20/12-20/3); Autumn (21/9-20/12); Summer (21/6-20/9); Spring (21/3-20/6).

Appendix B4

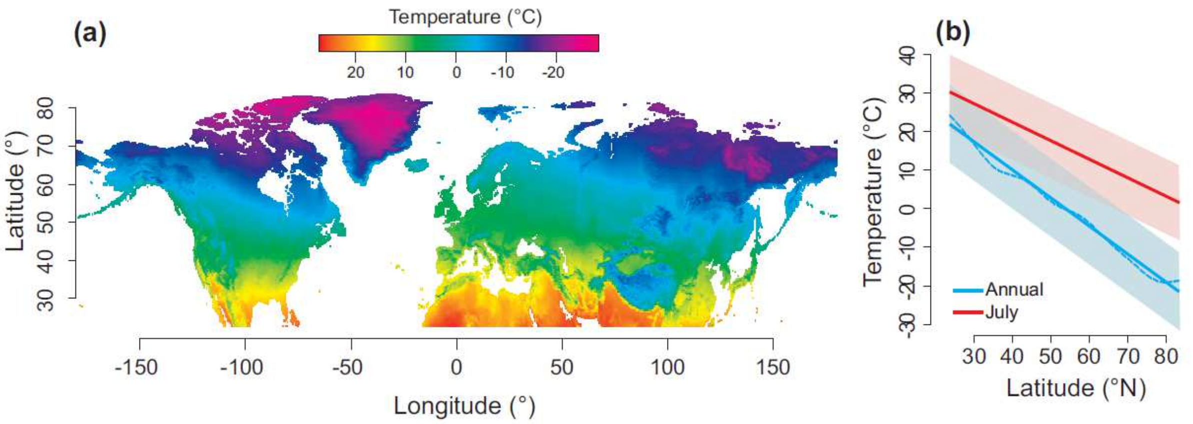

Fig. B4: The latitudinal temperature gradient in the Northern extratropical hemisphere. (a) Map of the mean annual temperature in the Northern extratropical hemisphere. (b) Relationships between latitude and mean annual and July temperatures (De Frenne *et al.* 2013).

Appendix B5

Table B5. Pearson's correlation between three environmental variables. All the correlations were non significant (p-value >0.05), except the correlation between potentially acidifying deposition and nitrogen deposition (***), which had a $p < 0.001$ ($n = 39$).

	Growing degree days year before sampling	Potentially acidifying deposition	Nitrogen deposition
Potentially acidifying deposition	0.222		
Nitrogen deposition	0.222	0.988***	
Annual precipiation	-0.301	0.126	0.103

Appendix C

Table C1. Population characteristics (part one)

Population number	Country	Latitude (°)	Longitude (°)	Elevation (m above sea level)	Number of sampled shrubs	Number of sampled SP2 seeds	Number of sampled SP3 seeds	Soil texture	Type of bedrock
1	Spain	41.79	-2.84	1240	5	176	52	loamy	calcareous
2	Italy	43.23	11.18	320	5	173	124	sandy loam	calcareous
3	France	43.26	0.87	300	5	167	122	clayey	calcareous
4	France	44.86	5.48	1500	5	224	9	loamy	calcareous
5	Italy	45.87	11.65	619	4	145	60	sandy loam	calcareous
6	Italy	45.89	11.47	1095	5	163	99	sandy loam	calcareous
7	Switzerland	46.86	9.23	2128	3	69	63	sandy	calcareous
8	Austria	47.27	11.27	805	5	105	159	sandy	calcareous
9	Germany	49.97	11.31	450	5	163	109	sandy loam	calcareous
10	Belgium	50.1	5.19	345	5	173	116	loamy	non-calcareous
11	Germany	50.21	6.38	595	5	191	106	loamy	non-calcareous
12	Belgium	50.44	5.87	535	5	161	95	clayey	non-calcareous
13	Germany	50.76	8.32	419	5	174	122	sandy loam	non-calcareous
14	UK	51.23	-0.44	175	4	111	119	sandy loam	calcareous
15	Netherlands	51.55	6.01	50	5	175	112	sandy	non-calcareous
16	Germany	51.95	13.49	115	5	143	140	sandy	non-calcareous
17	Germany	52.93	9.35	40	5	162	130	sandy	non-calcareous
18	Sweden	55.67	13.37	70	4	114	101	sandy loam	non-calcareous
19	Sweden	56.54	16.47	45	5	157	128	sandy loam	calcareous
20	Sweden	59.18	18.38	4	5	165	97	sandy loam	non-calcareous

Table C2. Poplation characteristics (part two)

Population number	N-deposition (kg.ha^{-1}.year^{-1})	Potentially acidifying depositions (keq.ha^{-1}.year^{-1})	Precipitation (mm.year^{-1})	Temperature (GDD>0°C)	Weather station	Source weather data
1	5.66	0.54	699.3	3790	Soria	http://eca.knmi.nl/ (2)
2	9.29	0.94	906.1	5074.7	Siena Orto Botanico	http://www.biometeo.it
3	9.72	0.84	836.3	4877.6	Toulouse-Blagnac	http://eca.knmi.nl/ (2)
4	10.44	0.97	1211.4	2315.9	IIserech2	http://eca.knmi.nl/ (2)
5	28.13	2.38	1076.4	4461.2	Pove del Grappa loc. Pra' Gollin	http://www.arpa.veneto.it (4)
6	20.09	1.69	1461	2598.2	Asiago (aeroporto)	http://www.arpa.veneto.it (4)
7	8.7	0.76	1693.4	1275.1	Crap Masegn - Schneestation	http://www.slf.ch/ (3)
8	7.81	0.69	1605.2	3324.3	Innsbruck-Flughafen	ZAMG Tirol und Vorarlberg
9	14.42	1.37	909.8	3049.4	Bamberg	http://www.dwd.de/
10	14.97	1.46	949.9	3353.6	Humain	http://www.kmi.be
11	15.88	1.63	1004.4	2897.8	Schneifelforsthaus	http://www.dwd.de/
12	17.62	1.76	902.8	2970.5	Mont-Rigi	http://www.kmi.be
13	13.62	1.4	907.5	3170	Dillenburg	http://www.dwd.de/
14	11.2	1.12	829.9	3494	Mickleham	http://www.metoffice.gov.uk/
15	25.25	2.28	829.1	3703.7	Arcen	http://eca.knmi.nl/ (2)
16	13.25	1.32	726.6	3292.3	Baruth	http://www.dwd.de/
17	18.61	1.73	846	3375.3	Rotenburg	http://www.dwd.de/
18	9.21	0.95	804.4	3217.3	Lund	http://www.smhi.se/
19	5.06	0.61	652.3	NA	Ölandsbron	http://www.smhi.se/
20	4.85	0.51	710.4	3102.3	Stockholm	http://www.smhi.se/

NA: data not available

Appendix D1

Table D1. Information on the origin of the shrubs. Population names and information are acquired from Gruwez et al. (2010a). If more than one shrub was originating from the same mother shrub, this was denoted with clx (x being the code of the moder shrub).

Site	Shrub code	Gender	Poplation	Number of shurbs in population	Clone
Sweden	St.1	female	Kattevennen	820	cl2
Sweden	St.2	female	Heiderbos	7000	
Sweden	St.3	female	32	4	
Sweden	St.4	female	84	1	cl9
Sweden	St.5	female	Heiderbos	7000	
Sweden	St.6	female	Kattevennen	820	cl1
Sweden	St.7	female	Kattevennen	820	cl7
Sweden	St.8	male	Heiderbos	7000	
Sweden	St.9	male	Kattevennen	820	
Belgium high elevation	Hv.1	female	Heiderbos	7000	
Belgium high elevation	Hv.2	female	84	1	cl9
Belgium high elevation	Hv.3	female	Kattevennen	820	cl3
Belgium high elevation	Hv.4	female	Kattevennen	820	cl4
Belgium high elevation	Hv.5	female	Kattevennen	820	cl5
Belgium high elevation	Hv.6	female	Kattevennen	820	cl1
Belgium high elevation	Hv.7	female	Zutendaal	129	cl8
Belgium high elevation	Hv.8	male	Heiderbos	7000	
Belgium high elevation	Hv.9	male	Kattevennen	820	cl6
Belgium low elevation	Go.1	female	Zutendaal	129	
Belgium low elevation	Go.2	female	Heiderbos	7000	
Belgium low elevation	Go.3	female	Zutendaal	129	
Belgium low elevation	Go.4	female	Zutendaal	129	cl8
Belgium low elevation	Go.5	female	84	1	cl9
Belgium low elevation	Go.6	female	Kattevennen	820	cl7
Belgium low elevation	Go.7	female	Kattevennen	820	cl5
Belgium low elevation	Go.8	male	Kattevennen	820	cl6
Belgium low elevation	Go.9	male	Zutendaal	129	
France	To.1	female	Kattevennen	820	cl3
France	To.2	female	45J	11	
France	To.3	female	20	8	
France	To.4	female	25	5	
France	To.5	female	Zutendaal	129	
France	To.6	female	Heiderbos	7000	
France	To.7	female	Kattevennen	820	cl4
France	To.8	male	Kattevennen	820	cl6
France	To.9	male	Zutendaal	129	
Open top chamber	GS.1	female	Kattevennen	820	cl7
Open top chamber	GS.2	female	Heiderbos	7000	
Open top chamber	GS.3	female	Zutendaal	129	
Open top chamber	GS.4	female	Kattevennen	820	cl2

Site	Shrub code	Gender	Poplation	Number of shurbs in population	Clone
Open top chamber	GS.5	female	Kattevennen	820	cl1
Open top chamber	GS.6	female	84	1	cl9
Open top chamber	GS.7	female	7	8	
Open top chamber	GS.8	male	Zutendaal	129	
Open top chamber	GS.9	male	Heiderbos	7000	

Appendix D2

Table D2. The minimum, maximum, and mean (± SE) percentages viable seeds per shrub for the whole experiment (three years) and for each year separately.

	Seed phase 2 seeds			Seed phase 3 seeds		
	Min	Max	Mean ± SE	Min	Max	Mean ± SE
Total	15.8	100.0	84.1 ± 1.8	0.0	100.0	60.6 ± 3.3
2011	67.6	100.0	92.4 ± 1.6	0.0	100.0	42.8 ± 6.7
2012	48.1	100.0	86.1 ± 2.6	50.0	100.0	77.0 ± 2.6
2013	15.8	100.0	74.5 ± 3.8	0.0	100.0	59.1 ± 5.9

Appendix D3

Table D3. The average monthly minimum and maximum temperatures (°C) at the four sites of the transplant experiment during the runtime of the experiment.

		Average monthly minimum temperature (°C)				Average monthly maximum temperature (°C)			
Month	Year	Central Sweden	Belgium, high elevation	Belgium, low elevation	Southern France	Central Sweden	Belgium, high elevation	Belgium, low elevation	Southern France
11	2011	1.5	3.1	5.2	8.5	4.9	10.0	11.8	15.7
12	2011	-2.1	-0.4	4.6	3.9	1.4	3.8	8.6	11.9
1	2012	-5.1	-1.0	3.5	2.7	-1.4	2.9	7.8	10.2
2	2012	-10.0	-6.0	-2.0	-3.5	-1.5	-1.0	4.2	6.0
3	2012	-2.3	2.3	4.2	3.1	6.0	10.1	13.3	17.3
4	2012	-1.5	1.2	4.5	6.6	8.2	8.8	12.6	16.0
5	2012	4.2	6.9	9.9	10.5	17.5	16.1	18.9	21.8
6	2012	7.7	8.2	12.3	13.9	18.0	15.8	19.7	26.1
7	2012	11.9	9.8	13.4	13.6	23.9	18.2	21.9	27.0
8	2012	11.0	10.7	13.7	16.3	21.0	20.4	24.4	30.0
9	2012	7.0	6.5	10.1	12.8	13.4	15.0	19.7	24.4
10	2012	1.5	4.5	8.2	10.0	6.7	11.3	15.1	20.2
11	2012	0.1	1.3	5.4	5.8	3.3	5.7	9.9	13.8
12	2012	-6.9	-1.0	3.9	3.6	-3.7	2.4	7.4	11.5
1	2013	-9.5	-3.3	0.0	1.4	-3.9	-0.1	4.0	8.4
2	2013	-6.7	-4.8	0.1	0.9	-0.4	-1.3	4.3	9.0
3	2013	-12.5	-4.3	0.2	5.1	1.7	1.4	6.4	13.7
4	2013	-3.2	1.0	4.3	6.3	9.4	9.0	13.6	15.9
5	2013	6.0	4.4	7.6	8.2	17.5	12.0	15.4	17.4
6	2013	9.7	7.9	11.4	11.8	22.6	16.4	20.2	22.1
7	2013	10.9	12.1	14.1	16.3	22.5	21.5	25.0	30.4
8	2013	10.1	9.7	13.5	15.0	23.4	19.4	23.7	27.7
9	2013	8.2	7.7	10.8	13.2	16.6	15.4	19.5	24.3
10	2013	NA	6.4	9.8	11.6	NA	12.0	16.7	21.0
11	2013	NA	0.4	4.6	4.9	NA	4.4	9.4	11.3

NA: no data available

References

Adams RP. 2008. Junipers of the World: the genus Juniperus, 2nd edn. Vancouver, Trafford Publishing.

Adriaenssens S. 2006. Vergelijkend onderzoek naar de productie en kiemkracht van jeneverbeszaden in Vlaanderen en omliggende regio's. MSc thesis, Genth, Ghent University.

Adriaenssens S, Baeten L, Crabbe S, Verheyen K. 2006. Evolutie (1985-2006) en toekomst van de jeneverbes (Juniperus communis L.) in de provincie Limburg. Ghent, Ghent University & Likona.

Aerts R, Boot RGA, Van der Aart PJM. 1991. The relation between above- and belowground biomass allocation patterns and competitive ability. Oecologia 87: 551–559.

Aerts R, Heil GW. 1993. Heathlands: patterns and processes in a changing environment. Dordrecht, Kluwer.

Aerts R, Chapin FS. 2000. The mineral nutrition of wild plants revisited: a reevaluation of processes and patterns. Advances in Ecological Research 30: 1-67.

Anderson ED, Owens JN. 2000. Microsporogenesis, pollination, pollen germination and male gametophyte development in *Taxus brevifolia*. Annals of Botany 86: 1033-1042.

Alonso I. 2004. La conservación de los brezales en Inglaterra. Ecosistemas 13: 1-9.

Augustin S, Bolte A, Holzhausen M, Wolff B. 2005. Exceedance of critical loads of nitrogen and sulphur and its relation to forest conditions. European Journal of Forest Research 124: 289-300.

Azevedo LB, van Zelm R, Hendriks AJ, Bobbink R, Huijbregts MAJ. 2013. Global assessment of the effects of terrestrial acidification on plant species richness. Environmental Pollution 174: 10-15.

Baeten L, Vanhellemont M, De Frenne P, Hermy M, Verheyen K. 2010. The phosphorus legacy of former agricultural land use can affect the production of germinable seeds in forest herbs. Ecoscience 17: 365–371.

Bakker A. 1988. Gymnosporangium bij *Juniperus communis* L. en het verband tussen taksterfte in de toppen van Juniperus communis en het VAM-percentage. PhD thesis, Wageningen, Wageningen Univerisity.

Barkman JJ. 1989. Syllabus caput selectum: Nederlandse Boomsoorten II. Vakgroep Bosteelt en Bosoecologie 1989. *Juniperus communis* L.

Bascomb CL. 1964. Rapid method for the determination of cation-exchange capacity of calcareous and non-calcareous soils. Journal of the Science of Food and Agriculture 15: 821-823.

Bergman L. .1963. De natuurlijke verjonging van Juniperus communis (jeneverbes) in Nederland. In opdracht van het Nationale Park “De Hoge Veluwe”. Scriptie R.I.V.O.N.

Bishnoi UR, Kaur G, Khan MH. 2007. Calcium, Phosphorus, and Harvest Stages Effects Soybean Seed Production and Quality. Journal of Plant Nutrition 30: 2119-2127.

Blanes MC, Viñegla B, Merino J, Carreira JA. 2013. Nutritional status of Abies pinsapo forests along a nitrogen deposition gradient: do C/N/P stoichiometric shifts modify photosynthetic nutrient use efficiency? Oecologia 171: 797-808.

Bobbink R, Jeil GW, Raessen MBAG. 1992. Atmospheric deposition and canopy exchange processes in heathland ecosystems. Environmental Pollution 75: 29-37.

Bobbink R, Hornung M, Roelofs JGM. 1998. The effects of air-borne pollutants on species diversity in natural and semi-natural European vegetation. Journal of Ecology 86: 717-738.

Bobbink R, K Hicks, J Galloway, T Spranger, R Alkemade, M Ashmore, M Bustamante, S Cinderby, E Davidson, F Dentener, B Emmett, J-W Erisman, M Fenn, F Gilliam, A Nordin, L Pardo and W de Vries. 2010. Global assessment of nitrogen deposition effects on terrestrial plant diversity effects of terrestrial ecosystems: a synthesis. Ecological Applications 20: 30-59.

Bolker BM. 2008. Ecological models and data. Princeton, Princeton University Press.

Bonner F.T. (2008) Seed biology. In Bonner F.T., Karrfalt R.P. (Eds). Woody Plant Seed Manual. Washington DC, US Department of Agriculture Forest Service: 3-38.

Bowman WD, Cleveland CC, Halada L, Hresko J. 2008. Negative impact of nitrogen deposition on soil buffering capacity. Nature Geoscience 1: 767–770.

Breek J. 1978. De kiemingsecologie van *Juniperus communis* L. MSc thesis, Utrecht, Utrecht University.

Broome A. 2003. Growing juniper: propagation and establishment practices. Forestry Commission Information Note 50. Edinburgh, Forestry Commission.

Burnham KP, Anderson DR. 2002. Model selection and multimodel inference: A practical information-theoretic approach. New York, Springer.

Burny J. 1985.het vroeger en huidig voorkomen van jeneverbes *Juniperus communis* L. op de Hoge Kempen (provincie Limburg, België). Wielewaal 5: 10–30.

Callahan HS, Del Fierro K, Patterson AE, Zafar H. 2008. Impacts of elevated nitrogen inputs on oak reproductive and seed ecology. Global Change Biology 14: 285–293.

Chambers JC, Schupp EW, Vander Wall SB. 1999. Seed and seedling ecology of pińon and juniper species. The Botanical Review 65: 1-38.

Charles DJ. 2013. Antioxidant properties of spices, herbs and other sources. New York, Springer.

Clark CM, Tilman D. 2008. Loss of plant species after chronic low-level nitrogen deposition to prairie grasslands. Nature 451: 712-715.

Clifton SJ, Ward LK, Ranner DS. 1997. The status of Juniper *Juniperus communis* L. in North-East England. Biological Conservation 79: 67-77.

Cross RH, McKay SAB, McHughen AG, Bonham-Smith PC. 2003. Heat-stress effects on reproduction and seed set in *Linum usitatissimum* L. (flax). Plant, Cell and Environment 26: 1013-1020.

De Bakker H. 1979. Major soils and soil regions of The Netherlands. Wageningen, Junk, Den Haag and Pudoc.

De Frenne P, De Schrijver A, Graae BJ, Gruwez R, Tack W, Vandelook F, Hermy M, Verheyen K. 2010. The use of open-top chambers in forests for evaluating warming effects on herbaceous understory plants. Ecological Research 25: 163-171.

De Frenne P, Brunet J, Shevtsova A, Kolb A, Graae BJ, Chabrerie O, Cousins SAO, Decocq G, De Schrijver A, Diekman M, Gruwez R, Heinken T, Hermy M, Nilsson C, Stanton S, Tack W, Willaert J, Verheyen K. 2011. Temperature effects on forest herbs assessed by warming and transplant experiments along a latitudinal gradient. Global Change Biology 17: 3240-3253.

De Frenne P, Graae BJ, Rodríguez-Sánchez F, Kolb A, Chabrerie O, Decocq G, De Kort H, De Schrijver A, Diekmann M, Eriksson O, Gruwez R, Hermy M, Lenoir J, Plue J, Coomes DA, Verheyen K. 2013. Latitudinal gradients as natural laboratories to infer species' responses to temperature. Journal of Ecology 101: 784-795.

De Graaf M, Bobbink R, Roelofs JGM, Verbeek JM. 1998. Differential effects of ammonium and nitrate on three heathland species. Plant Ecology 135: 185–196.

DeLuca TH, Zackrisson O. 2007. Enhanced soil fertility under *Juniperus communis* in arctic ecosystems. Plant Soil 294: 147-155.

Demirtas C, Yazgan S, Candogan BN, Sincik M, Buyukcangaz H, Goksoy AT. 2010. Quality and yield response of soybean (*Glycine max* L. Merrill) to drought stress in sub–humid environment. African Journal of Biotechnology 9: 6873-6881.

Despland E, Houle G. 1997. Climate influences on growth and reproduction of *Pinus banksiana* (Pinaceae) at the limit of the species distribution in Eastern North America. American Journal of Botany 84: 928-937.

De Schrijver A, De Frenne P, Ampoorter E, Van Nevel L, Demey A, Wuyts K, Verheyen K. 2011. Cumulative nitrogen input drives species loss in terrestrial ecosystems. Global Ecology Biogeography 20: 803-816.

Diffenbaugh NS, Field CB. 2013. Changes in ecologically critical terrestrial climate conditions. Science 341: 486-492.

Doiron M, Gauthier G, Lévesque E. 2014. Effects of experimental warming on nitrogen concentration and biomass of forage plants for an arctic herbivore. Journal of Ecology 102: 508-517.

Dorland E, van den Berg LJL, van de Berg AJ, Vermeer ML, Roelofs JGM, Bobbink R. 2004. The effects of sod cutting and additional liming on potential net nitrification in heathland soils. Plant Soil 265:267–277.

Drenovsky RE, Richards JH. 2005. Nitrogen addition increases fecundity in the desert shrub *Sarcobatus vermiculatus*. Oecologia 143: 349-356.

Drews GN, Koltunow AMG. 2011. The Female Gametophyte. The Arabidopsis Book. 9: e0155, doi:10.1199/tab.0155.

Duquesnay A, Dupouley JL, Clement A, Ulrich E, Le Tacon F. 2000. Spatial and temporal variability of foliar mineral concentration in beech (*Fagus sylvatica*) stands in northeastern France. Tree Physiol 20: 13-22.

Durzan DJ, Chalupa V. 1968. Free sugars, amino acids, and soluble proteins in the embryo and female gametophyte of jack pine as related to climate and seed source. Canadian Journal of Botany 46: 417-428.

Durzan DJ. 2002. Stress-induced nitric oxide and adaptive plasticity in conifers. Journal of Forest Science 48: 281-291.

Falinski JB. 1980. Vegetation dynamics and sex structure of the populations of pioneer dioecious woody plants. Vegetatio 43: 23-38.

F.A.O. 2005. NewLocClim. Vers. 1.10. Rome, Food and Agriculture Organisation of the United Nations.

Fenner M, Thompson K. 2005. The ecology of seeds. Cambridge, Cambridge University Press.

Feret PP, Diebel KE, Sharik TL. 1990. Effect of simulated acid rain on reproductive attributes of red spruce (*Picea rubens* Sarg.). Environmental and Experimental Botany 30: 309-312.

Fernando DD, Owens JN, von Anderkas P, Takaso T. 1997. In vitro pollen tube growth and penetration of female gametophyte in Douglas fir (*Pseudotsuga menziesii*). Sexual Plant Reproduction 10: 209-216.

Fernando DD, Lazzaro MD, Owens JN. 2005. Growth and development of conifer pollen tubes. Sexual plant reproduction 18: 149-162.

Fitter AH, Jennings RD. 1975. The effects of sheep grazing on the growth and survival of seedling junipers (*Juniperus communis* L.). Journal of Applied Ecology 12: 637–642.

Fitter AH, Hay RKM. 2002. Environmental physiology of plants. London, Academic Press.

Forbes ARD, Proctor J. 1986. The Glen Artney juniper wood. Transactions of the Botanical Society of Edinburgh 45: 63–72.

Frankard P. 2004. Evolution de la population de *Juniperus communis* L. dans la réserve naturelle domaniale de la genévrière de Cour pendant ces vingt dernières années et impact des mesures de gestion appliquées. Parcs et Réserves 59: 32-37.

Franz RE, Jolliff GD. 1989. Temperature Effects on Megagametophytic Development in Meadowfoam. Crop Science 29: 133-141.

Fridley JD. 2009. Downscaling Climate over Complex Terrain: High Finescale (<1000 m) Spatial Variation of Near-Ground Temperatures in a Montane Forested Landscape (Great Smoky Mountains). Journal of applied meteorology and climatology 48: 1033-1049.

Galloway JN, Dentener FJ, Capone DG, Boyer EW, Howarth RW, Seitzinger SP, Asner GP, Cleveland C, Green P, Holland E, Karl DM, Michaels AF, Porter JH, Townsend A, Vörösmarty C. 2004. Nitrogen Cycles: Past, Present and Future. Biogeochemistry 70: 153-226.

García D. 1998. Interaction between juniper *Juniperus communis* L. and its fruit insect pests: pest abundance, fruit characteristics and seed viability. Acta Oecologica 19: 517– 525.

García D, Zamora R, Gómez JM, Hódar JA, Jordano P. 1999. Age structure of *Juniperus communis* L. in the Iberian peninsula: Conservation of remnant populations in Mediterranean Mountains. Biological conservation 87: 215-220.

García D, Zamora R, Gómez JM, Jordano P, Hódar JA. 2000. Geographical variation in seed production, predation and abortion in *Juniperus communis* throughout its range in Europe. Journal of Ecology 88: 436–446.

García D. 2001. Effects of seed dispersal on *Juniperus communis* on a Mediterranean mountain. Journal of Vegetation Science 12: 839-848.

García D, Zamora R, Gómez JM, Hódar JA. 2002. Annual variability in reproduction of *Juniperus communis* L. in a Mediterranean mountain: Relationship to seed predation and weather. Écoscience 9: 251-255.

Garg N, Chandel S. 2010. Arbuscular mycorrhizal networks: process and functions. A review. Agronomy for Sustainable Development 30: 581-599.

Gifford E, Foster A. 1989. Morphology and Evolution of Vascular Plants, 3rd edn. New Yord, W.H. Freeman.

Gilbert OL. 1980. Juniper in Upper Teesdale. Journal of Ecology 68: 1013–1024.

Gordon D.R. (1996). Experimental translocation of the endangered shrub *Apalachicola rosemary Conradina glabra* to the Apalachicola Bluffs and Ravines Preserve, Florida. Biological Conservation 77: 19-26.

Grubb PJ, Lee WG, Kollmann J, Wilson JB. 1996. Interaction of irradiance and soil nutrient supply on growth of seedlings of ten European tall-Shrub species and *Fagus Sylvatica*. Journal of Ecology 84: 827-840.

Gruwez R, Vanden Broeck A, Verheyen K. 2010a. Studie voor de opmaak van een soortbeschermingsplan voor jeneverbes (*Juniperus communis* L.) in Vlaanderen. Gent, Universiteit Gent & INBO.

Gruwez R, Vanden Broeck A, De Frenne P, Tack W, Verheyen K. 2010b. Welke toekomst voor de jeneverbes in Vlaanderen: een evaluatie aan de hand van beheerscenario's. Natuur.Focus 9: 167-173.

Hall B, Motzkin G, Foster DR, Syfert M, Burk J. 2002. Three hundred years of forest and land-use change in Massachusetts, USA. Journal of Biogeography 29: 1319–1335.

Han W, Fang J, Guo D, Zhang Y. 2005. Leaf nitrogen and phosphorus stoichiometry across 753 terrestrial plant species in China. New Phytologist 168: 377-385.

Harris I, Jones PD, Osborn TJ, Lister DH. 2013. Updated high-resolution grids of monthly climatic observations – the CRU TS3.10 Dataset. International Journal of Climatology 34: 623-642.

Hedhly A, Hormaza JI, Herrero M. 2003. The effect of temperature on stigmatic receptivity in sweet cherry (*Prunus avium* L.). Plant, Cell and Environment 26: 1673-1680.

Hedhly A, Hormaza JI, Herrero M. 2004. Effect of temperature on pollen tube kinetics and dynamics in sweet cherry, *Prunus avium* (Rocaceae). American Journal of Botany 91: 558-564.

Hedhly A, Hormaza JI, Herrero M. 2005. The effect of temperature on pollen germination, pollen tube growth, and stigmatic receptivity in Peach. Plant Biology 7: 476-483.

Hedhly A, Hormaza JI, Herrero M. 2009. Global warming and sexual plant reproduction. Trends in plant science 14: 30-36.

Hedhly A. 2011. Sensitivity of flowering plant gametophytes to temperature fluctuations. Environmental and experimental botany 74: 9-16.

Henry DG. 1973. Foliar nutrient concentrations of some Minnesota forest species. Minnesota Forestry Research Notes No. 241. Minnesota, College of Forestry, University of Minnesota.

Herrero M. .2003. Male and female synchrony and the regulation of mating in flowering plants. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 358: 1019–1024.

Hikosaka K. 1997. Modelling optimal temperature acclimation of the photosynthetic apparatus in C_3 plants with respect to nitrogen use. Annals of Botany 80: 721-730.

HilleRisLambers J, Harpole WS, Schnitzer S, Tilman D, Reich PB. 2009. CO2, nitrogen, and diversity differentially affect seed production of prairie plants. Ecology 90: 1810-1820.

Hobbie SE, Gough L. 2002. Foliar and soil nutrients in tundra on glacial landscapes of contrasting ages in northern Alaska. Oecologia 131: 453-462.

Hof C, Levinsky I, Araújo MB, Rahbek C. 2011. Rethinking species' ability to cope with rapid climate change. Global Change Biology 17: 2987-2990.

Hommel PWFM, Griek M, Haveman R, den Ouden J, de Waal RW. 2009. Herstel van jeneverbesstruwelen. De Levende Natuur 110:135–137.

Hovenden MJ, Wills KE, Chaplin RE, Vander Schoor JK, Williams AL, Osanai Y, Newton PCD. 2008. Warming and elevated CO2 affect the relationship between seed mass, germinability and seedling growth in *Austrodanthonia caespitosa*, a dominant Australian grass. Global Change Biology 14: 1-9.

Hultén E, Fries M. 1986. Atlas of North European vascular plants: North of the Tropic of Cancer I-III. Königstein, Koeltz Scientific Books.

Hüppe J. 1995. Zur Problematik der Verjüngung des Wacholders (Juniperus communis) unter dem Einfluβ von Wildkaninchen in Hudegebieten pleistozäner Sandlandschaften. Zeitschrift für Ökologie und Naturschutz 4: 1-8.

Hurvich CM, Tsai CL. 1989. Regression and time series model selection in small samples. Biometrika 76: 297-307.

Innes JL. 1995. Influence of air pollution on the foliar nutrition of conifers in Great Britain. Environmental Pollution 88: 183-192

IPCC. 2013. Climate change 2013: the physical science basis. Cambridge, Cambridge University Press.

Izuta T, Yamaoka T, Nakaji T, Yonekura T, Yokoyama M, Funada R, Koike T, Totsuka T. 2004. Growth, net photosynthesis and leaf nutrient status of *Fagus crenata* seedlings grown in brown forest soil acidified with H2SO4 or HNO3 solution. Trees 18: 677-685.

Jett JB. 1987. Seed orchard management: something old and something new. In Proc. 19th South. For. Tree Improv. Conf. College Station, TX, pp 160--171

Kang H, Zhuang H, Wu L, Liu Q, Shen G, Berg B, Man R, Liu C. 2011. Variation in leaf nitrogen and phosphorus stoichiometry in Picea abies across Europe: An analysis based on local observations. Forest Ecol Manag 261: 195-202.

Kärkkäinen K, Savolainen O, Koski V. 1999. Why do plants abort so many developping seeds: bad offspring or bad maternal genotypes? Evolutionary Ecology 13: 305-317.

Karlsson C, Örlander G. 2002. Mineral nutrients in needles of *Pinus sylvestris* seed trees after release cutting and their correlations with cone production and seed weight. Forest Ecology and Management 166: 183-191

Karlsson C. 2006. Fertilization and release cutting increase seed production and stem diameter growth in *Pinus sylvestris* seed trees. Scandinavian Journal of Forest Research 21: 317-326.

Kelly JK, Rasch A, Kalisz S. 2002. A method to estimate pollen viability from pollen size variation. American Journal of Botany 89: 1021-1023.

Knol WC, Nijhof BSJ. 2004. Jeneverbes (*Juniperus communis* L.) in de verdrukking. Een integrale verkenning van de verjongingsproblematiek. Wageningen, Alterra.

Koivuranta L, Latva-Karnanmaa T, Pulkkinen P. 2012. The Effect of Temperature on Seed Quality and Quantity in Crosses between European (*Populus tremula*) and Hybrid Aspens (*P. tremula x P. tremuloides*). Sylva Fennica 46: 17-26.

Körner. 2007. The use of ‘altitude’ in ecological research. Trends in Ecology and Evolution 22: 569-574.

Kozai N, Beppu K, Mochioka R, Boonprakob U, Subhadradandhu S, Katoaka I. 2004. Adverse effects of high temperature on the development of reproductive organs in 'Hakuho' peach trees. Journal of Horticultural Science & Biotechnology 79: 533-537.

Krupa SV. 2003. Effects of atmospheric ammonia (NH3) on terrestrial vegetation: a review. Environmental Pollution 124: 179-221.

Kurz ML, Roberts DR, Flinn BS. 1994. A morphological and biochemical analysis of embryo maturation during yellow cypress seed development. Canadian Journal of Forest Research 24: 431-441.

Lajtha K, Driscoll CT, Jarrell WM, Elliott ET. 1999. Soil phosphorus: characterization and total element analysis. In: Robertson GP, Coleman DC, Bledsoe CS, Sollins P (eds). Standard soil methods for long-term ecological research. New York, Oxford University Press: 115–142.

Leroux O, Van der Kinderen G, Viane RLL. 2007. A sandwich-embedding method for oriented sectioning. Journal of Microscopy 227: 79-82.

Li Y, Yang H, Xia J, Zhang W, Wan S, Li L. 2011. Effects of Increased Nitrogen Deposition and Precipitation on Seed and Seedling Production of *Potentilla tanacetifolia* in a Temperate Steppe Ecosystem. PLoS ONE 6: e28601. doi:10.1371/journal.pone.0028601.

Linkies A, Graeber K, Knight C, Leubner-Metzger G. 2010. The evolution of seeds. New Phytologist 186: 817-831.

Livingston RB. 1972. Influence of birds, stones and soil on the establishment of pasture juniper, *Juniperus communis*, and red cedar, *J. virginiana* in New England pastures. Ecology 53: 1141–1147.

Lucassen E, Loeffen L, Popma J, Verbaarschot E, Remke E, de Kort J, Roelofs J. 2011. Bodemverzuring lijkt een sleutelrol te spelen in het verstoorde verjongingsproces van Jeneverbes. De Levende Natuur 6: 235-239.

Maes B, Bastiaens J, Brinkkemper O, Rövekamp C, Van den Bremt P, Zwaenepoel A. 2006. Inheemse bomen en struiken in Nederland en Vlaanderen. Amsterdam, Uitgeverij Boom.

Malcová R, Vosátka M, Albrechtová J. 1999. Influence of arbuscular mycorrhizal fungi and simulated acid rain on the growth and coexistence of the grasses *Calamagrostis villosa* and *Deschampsia flexuosa*. Plant and Soil 207: 45-57.

Marcott SA, Shakun JD, Clark PU, Mix AC. 2013. A reconstruction of regional and global temperature for the past 11,300 years. Science 339: 1198-1201.

Marschner H. 1995. Mineral Nutrition of Higher Plants. 2nd Edn. London, Academic Press.

Matesanz S, Gianoli E, Valladares F. 2010. Global change and the evolution of phenotypic plasticity in plants. Annals of the New York Academy of Sciences 1206: 35-55.

McVean DN. 1966. Establishment of native trees and shrubs on Scottish nature reserves by direct seed sowing. Scottish Forestry 20: 26-36.

Menges ES. 2008. Restoration demography and genetics of plants: when is a translocation successful? Australian journal of botany 56: 187-196.

Meunier C, Sirois L, Bégin Y. 2007. Climate and *Picea mariana* seed maturation relationships: a multiple-scale perspective. Ecological Monographs 77: 361-376.

Michalczyk IM, Opgenoorth L, Luecke Y, Huck S, Ziegenhagen B. 2010. Genetic support for perglacial survival of *Juniperus communis* L. in Central Europe. Holocene 20: 887-894.

Millennium Ecosystem Assessment (MEA). 2005. Ecosystems and Human Well-Being: Scenarios. Washington DC, Island Press.

Miller AL. 2004. Tetrazolium testing for flower seeds. In Mcdonald MB, Kwong FY (eds.). Flower seeds: Biology and Technology. Wallingford, Cabi Publishing: 229-310.

Mugnaini S, Nepi M, Guarnieri M, Piotto B, Pacini E. 2007. Pollination Drop in *Juniperus communis*: Response to Deposited Material. Annals of Botany 100: 1475-1481.

Munzuroglu O, Obek E, Geckil H. 2003. Effects of simulated acid rain on the pollen germination and pollen tube growth of apple. (*Malus sylvestris* Miller cv. Golden). Acta Biologica Hungarica 54: 95-103.

Nepi M, Guarnieri M, Mugnaini S, Cresti L, Pacini E., Piotto B. 2005. A modified FCR test to evaluate pollen viability in *Juniperus communis* L. Grana 44: 148–151.

Newton AC, Stewart GB, Myers G, Diaz A, Lake S, Bullock JM, Pullin AS. 2009. Impacts of grazing on lowland heathland in north-west Europe. Biological Conservation 142: 935-947.

Niemeyer M, Niemeyer T, Fottner S, Härdtle W, Mohamed A. 2007. Impact of sod-cutting and choppering on nutrient budgets of dry heathlands. Biological Conservation 137: 344–353.

Noland TL, Parker WC, Morneault AE. 2006. Natural variation in seed characteristics of eastern white pine (*Pinus stobus* L.). New Forests 32: 87-103.

Oostermeijer JGB, De Knegt B. 2004. Genetic population structure of the wind-pollinated, dioecious shrub *Juniperus communis* in fragmented Dutch heathlands. Plant Species Biology 19: 175-184.

Ottley AM. 1909. The development of the gametophytes and fertilization in *Juniperus communis* and *Juniperus virginia*. Botanical Gazette 48: 31-46.

Owens JN, Blake MD. 1986. Production de Semences Forestières. Rapport d'information PI-X-53F du Service Canadien des Forêts. Canada, Institut forestier national de Petawawa.

Owens JN. 1991. Flowering and seed set. In: Raghavendra AS (ed.). Physiology of Trees. New York, John Wiley: 247-271.

Owens JN. 1995. Constraints to seed production: temperate and tropical forest trees. Tree Physiology 15: 447–484.

Owens JN, Morris SJ. 1998. Factors affecting seed and cone development in Pacific sliver fir (*Abies amabilis*). Canadian journal of forest research 28: 1146 -1163.

Owens JN, Johnsen Ø, Dæhlen OG, Skrøppa T. 2001. Potential effects of temperature on early reproductive development and progeny performance in *Picea abies* (L.) Karst. Scandinavian Journal of Forest Research 16: 21–237.

Owens JN, Bennet J, L'Hirondelle S. 2005. Pollination and cone morphology affect cone and seed production in lodgepole pine seed orchards. Canadian journal of forest research 35: 383 -400.

Owens JN. 2006. The reproductive biology of lodgepole pine. Forest Genetics Council of BC, Victoria, BC. Ext. Note 07.

Owens JN, Kittirat T, Mahalovich MF. 2008. Whitebark pine (*Pinus albicaulis* Engelm.) seed production in natural stands. Forest Ecology and Management 255: 803–809.

Pack DA. 1921. After-ripening and germination of juniper seeds. Botanical Gazette 71:32–60.

Pearman PB, Randin CF, Broennimann O, Vittoz P, van der Knaap WO, Engler R, Le Lay G, Zimmermann NE, Guisan A. 2008. Prediction of plant species distributions across six millennia. Ecology Letters 11:357–369.

Pearson J, Stewart GR. 1993. The deposition of atmospheric ammonia and its effects on plants. New Phytologist 125: 283–305.

Peñuelas J, Gordon C, Llorens L, Nielsen T, Tietema A, Beier C, Bruna P, Emmett B, Estiarte M, Gorissen A. 2004. Nonintrusive field experiments show different plant responses to warming and drought among sites, seasons, and species in a north–south European gradient. Ecosystems 7: 598–612.

Piessens K, Honnay O, Nackaerts K, Hermy M. 2004. Plant species richness and composition of heathland relics in north-western Belgium: evidence for a rescue-effect? Journal of Biogeography 31: 1683–1692.

Piessens K, Honnay O, Hermy M. 2005. The role of fragment area and isolation in the conservation of heathland species. Biological Conservation 122: 61–69.

Piotto B, Bartolini G, Bussotti F, Calderón García AA, Chessa I, Ciccarese C, Ciccarese L, Crosti R, Cullum FJ, Di Noi A, García-Fayos P, Lambardi M, Lisci M, Lucci S, Melini S, Muñoz Reinoso JC, Murranca S, Nieddu G, Pacini E, Pagni G, Patumi M, Pérez García F, Piccini C, Rossetto M, Tranne G, Tylkowski T, 2003. Fact Sheets on the Propagation of Mediterranean Trees and Shrubs from Seed. In B Piotto, A Di Noi (eds.). Seed Propagation of Mediterranean Trees and Shrubs. Rome, APAT – Agency for the Protection of the Environment and for Technical Services: 11-51.

Quinn GP, Keough, MJ. 2002. Experimental Design and Data Analysis for Biologists. Cambridge, Cambridge University Press.

R Development Core Team. 2012. R: A Language and Environment for Statistical Computing. Vienna, R Foundation for Statistical Computing.

Rands MRW, Adams WM, Bennun L, Butchart SHM, Clements A, Coomes D, Entwistle A, Hodge I, Kapos V, Scharlemann JPW, Sutherland WJ, Vira B. 2010. Biodiversity Conservation: Challenges Beyond 2010. Science 329: 1298-1303.

Rautio P, Fürst A, Stefan K, Raitio H, Bartels U. 2010. Sampling and Analysis of Needles and Leaves. 19 pp. Manual Part XII. In: Manual on methods and criteria for harmonized sampling, assessment, monitoring and analysis of the effects of air pollution on forests, UNECE, ICP Forests Programme Co-ordinating Centre, Hamburg (http://www.icp-forests.org/Manual.htm)

Raven PH, Evert RF, Eichhorn SE. 1999. Biology of Plants, 6th ed. New York, WH Freeman and Company.

Reich PB, Oleksyn J. 2004. Global patterns of plant leaf N and P in relation to temperature and latitude. PNAS 101: 11001–11006.

Rodin LE, Bazilevich NI. 1967. Production and mineral cycling in terrestrial vegetation (translated by Scripta Technica Ltd). Edindurgh and London, Oiver and Boyd.

Roelofs JGM. 1986. The effect of airborne sulphur and nitrogen deposition on aquatic and terrestrial heathland vegetation. Experientia 42: 372–377.

Roques A, Skrzypczynska M. 2003. Seed-infesting chalcids of the genus *Megastigmus* Dalman, 1820 (Hymenoptera: Torymidae) native and introduced to the West Palearctic region: taxonomy, host specificity and distribution. Journal of Natural History 37: 127–238.

Rosén E. 1988. Development and seedling establishment within a *Juniperus communis* stand on Öland, Sweden. Acta Botanica Neerlandica 37: 193-201.

Rosén E. 1995. Periodic droughts and long-term dynamics of alvar grassland vegetation on Öland, Sweden. Folia Geobotanica Phytotaxonomica 30: 131–140.

Rosén E, Bakker JP. 2005. Effects of agri-environment schemes on scrub clearance, livestock grazing and plant diversity in a low-intensity farming system on Öland, Sweden. Basic and Applied Ecology 6: 195-204.

Runge M, Rode MW. 1991. Effects of soil acidity on plant associations. In: Ulrich B, Sumner ME (eds.). Soil Acidity. Berlin, Springer-Verlag: 183-202.

Rustad LE, Campbell JL, Marion GM, Norby RJ, Mitchell MJ, Hartley AE, Cornelissen JHC, Gurevitch J. 2001. A meta-analysis of the response of soil respiration, net nitrogen mineralization, and aboveground plant growth to experimental ecosystem warming. Oecologia 126: 543-562.

Saini HS, Sedgley M, Aspinall D. 1983. Effect of Heat Stress During Floral Development on Pollen Tube Growth and Ovary Anatomy in Wheat (*Triticum aestivum* L.). Australian Journal of Plant Physiology 10: 137–144.

Sardans J, Rivas-Ulbach A, Peñuelas J. 2011. Factors affecting nutrient concentration and stoichiometry of forest trees in Catalonia (NE Spain). Forest Ecology and Management 262: 2024-2034.

Savonen E-M, Saarsalmi A. 1999. Effects of clone and fertilization on the seed and foliar chemical composition of Scots pine (*Pinus sylvestris*) grafts. Silva Fenn 33: 107–117.

Schaberg PG, DeHayes DH, Hawley GJ. 2001. Anthropogenic Calcium Depletion: A Unique Threat to Forest Ecosystem Health? Ecosyst Health 7: 214-228.

Scheel KC. 1936. Colorimetric determination of phosphoric acid in fertilizerswith the Pulfrich photometer. Journal of Analytical Chemistry 105:256–269.

Scherrer D, Körner C. 2010. Infra-red thermometry of alpine landscapes challenges climatic warming projections. Global Change Biology 16: 2602-2613.

Shaffer ML. 1981. Minimum population sizes for species conservation. BioScience 31: 131-134.

Shahmier F, Ahmadi L, Mirza M, Koroi SAA. 2003. Secretory elements of needles and berries of *Juniperus communis* L. spp. *communis* and its volatile constituents. Flavour and Fragrance Journal 18: 425–428.

Singh H. 1978. Embryology of gymnosperms. Encyclopedia of Plant Anatomy, vol 10, pt. 2. Berlin, Gerbruder Borntraeger.

Soil Survey Staff. 1975. Soil Taxonomy – a basic system of soil classification for making and interpreting soil surveys. Agricultural Handbook 436. Washington DC, U.S. Department of Agriculture.

Steinacher G, Wagner J. 2012. Effects of temperature on the progamic phase in high-mountain plants. Plant Biology 14: 295-305.

Stevenson FJ, Cole MA. 1999. Cycles of Soil: Carbon, Nitrogen, Phosphorus, Sulfur, Micronutrients, 2nd edn. New York, Wiley-Interscience.

Stockmann GL. 1982. Resultaten van een populatieoecologisch (demografisch) onderzoek van de jeneverbes op enkele terreinen in Drenthe en Overijssel. Wageningen, Vakgroep vegetatiekunde, plantenoecologie en onkruidkunde, Landbouwhogeschool Wageningen.

Takaso T, Owens JN. 1996. Postpollination-prezygotic ovular secretions into the mycropylar canal in *Pseudotsuga menziesii* (Pinaceae). Journal of Plant Research. 109: 147-160.

Thimonier A, Pannatier EG, Schmitt M, Waldner P, Walthert L, Schleppi P, Dobbertin M, Kräuchi N. 2010. Does exceeding the critical loads for nitrogen alter nitrate leaching, the nutrient status of trees and their crown condition at Swiss Long-term Forest Ecosystem Research (LWF) sites? European Journal of Forest Research 129: 443-461.

Thomas CD, Cameron A, Green RE, Bakkenes M, Beaumont LJ, Collingham YC, Erasmus BFN, de Siqueira MF, Grainger A, Hannah L, Hughes L, Huntly B, van Jaarsveld AS, Midgley GF, Miles L, Ortega-Huerta MA, Peterson AT, Phillips OL, Williams SE. 2004. Extinction risk from climate change. Nature 427: 145-148.

Thomas PA, El-Barghathi M, Polwart A. 2007. Biological Flora of the British Isles: Juniperus communis L. Journal of Ecology 95: 1404-1440.

Thomé OW. 1885. Flora von Deutschland, Österreich und der Schweiz Gera, Germany.

Thurig B, Körner C, Stocklin J. 2003. Seed production and seed quality in a calcareous grassland in elevated CO2. Global Change Biology 9: 873–884.

Tilman D, Reich PB, Knops J, Wedin D, Mielke T, Lehman C. 2001. Diversity and Productivity in a Long-Term Grassland Experiment. Science 294: 843-845.

Ulrich B, Summer M.E. (eds.). Soil acidity. Berlin, Springer Verlag.

UN-ECE. 2003. Forest condition in Europe. Executive Report 2003. Federal Research Centre for Forestry and Forest Products (BFH), Geneva and Brussels, 2003, 42p. http://www.unece.org

Uren SC, Ainsworth N, Power SA, Cousins DA, Huxedurp LM, Ashmore MR. 1997. Long-term effects of ammonium sulphate on Calluna vulgaris. Journal of Applied Ecology 34: 208–216.

Van den Berg LJL, Vergeer P, Roelofs JGM. 2003. Heathland restoration in The Netherlands: Effects of turf cutting depth on germination of *Arnica montana*. Applied Vegetation Science 6: 117–124.

Vanden Broeck A, Gruwez R, Cox K, Adriaesnssens S, Michalczyk IM, Verheyen K. 2011. Genetic structure and seed-mediated dispersal rates of an endangered shrub in a fragmented landscape: a case study for Juniperus communis in northwestern Europe. BMC Genetics 12:73. doi: 10.1186/1471-2156-12-73.

Van Dijk P. 1982. Sexstructuur en reproductie in een aantal Nederlandse jeneverbesstruwelen. Doctoraal verslag, Groningen, Rijksuniversiteit Groningen.

Vanhaeren R. 1983. De positie van *Juniperus communis* in het staatsnatuurreservaat Heiderbos te As. De Groene Band 49: 2-21.

Vergeer P, Rengelink R, Ouborg NJ, Roelofs JGM. 2003. The effects of population size and genetic variation on the response of Succisa pratensis to eutrophication and acidification. Journal of Ecology 91: 600-609.

Verheyen K, Schreurs K, Vanholen B, Hermy M. 2005. Intensive management fails to promote recruitment in the last large population of *Juniperus communis* (L.) in Flanders (Belgium). Biological Conservation 124: 113-121.

Verheyen K, Adriaenssens S, Gruwez R, Michalczyk I, Ward L, Rosseel Y, Van den Broeck A, García D. 2009. *Juniperus communis*: victim of the combined action of climate change and nitrogen deposition? Plant Biology 11: 49-59.

Vuosku J, Sarjala T, Jokela A, Sutela S, Sääskilahti M, Suorsa M, Läärä E, Häggman H. 2009. One tissue, two fates: different roles of megagametophyte cells during Scots Pine embryogenesis. Journal of Experimental Botany 60: 1375-1386.

Walck JL, Hidayati SN, Dixon KW, Thompson K, Poschlod P. 2011. Climate change and plant regeneration from seed. Global Change Biology 17: 2145-2161.

Ward LK. 1973. The conservation of juniper. I. Present status of juniper in southern England. Journal of Applied Ecology 10: 165–188.

Ward LK. 1982. The conservation of juniper: longevity and old age. Journal of Applied Ecology 19: 917–928.

Ward L.K. 2007. Lifetime sexual dimorphism in *Juniperus communis* var. *Communis*. Plant Species Biology 22: 11-21.

Ward LK. 2010. Variation in ripening years of seed cones of Juniperus communis. Watsonia 28: 11-19.

Warren R, VanDerwal J, Price J, Welbergen JA, Atkinson I, Ramirez-Villegas J, Osborn TJ, Jarvis A, Shoo LP, Williams SE, Lowe J. 2013. Quantifying the benefit of early climate change mitigation in avoiding biodiversity loss. Nature Climate Change 3: 678-682.

Webb NR. 2002. Atlantic heathlands. In: Perrow MR, Davy AJ (eds.). Handbook of Ecological Restoration, Vol.2: Restoration in Practice. Cambridge, Cambridge University Press: 401–418.

Weeda EJ, Westra R, Westra C, Westra T. 1985. Nederlandse oecologische flora. Wilde planten en hun relaties 1. Hilversum, KNNV uitgeverij/IVN.

Weih M, Karlsson PS. 2001. Growth response of Mountain birch to air and soil temperature: is increasing leaf-nitrogen content an acclimation to lower air temperature? New Phytologist 150: 147-155.

Wertheim FS, Craker LE. 1987. Acid rain and pollen germination in corn. Environmental Pollution 48: 165-172.

White RE, Taylor AW. 1977. Effects of pH on phosphate adsorption and isotopic exchange in acid soils at low and high additions of soluble phosphate. Journal of Soil Science 28: 48–61.

Williams CG. 2009. Conifer Reproductive Biology. New York, Springer.

Willson MF, Burley N. 1983. Mate choice in plants: tactics, mechanisms, and consequences. Princeton, New Jersey, Princeton University Press.

Xu ZF, Hu TX, Wang KY, Zhang YB, Xian JR. 2009. Short-term responses of phenology, shoot growth and leaf traits of four alpine shrubs in a timberline ecotone to simulated global warming, Eastern Tibetan Plateau, China. Plant Species Biology 24: 27-34.

Young LW, Wilen RW, Bonham-Smith PC. 2004. High temperature stress of *Brassica napus* during flowering reduces micro- and megagametophyte fertility, induces fruit abortion, and disrupts seed production. Journal of experimental Botany 55: 485-495.

Yuan Z, Chen HYH. 2007. Global trends in senesced-leaf nitrogen and phosphorus. Global Ecology and Biogeography 18: 532-542.

Zeidler M, Banas M, Zenata M. 2009. Ecological conditions and the distribution of alpine juniper (*Juniperus communis* subsp *alpina*) in the HrubA1/2 Jesenik Mts. Biologia 64: 687-693.

Zheng S, Shangguan Z. 2007. Spatial patterns of leaf nutrient traits of the plants in the Loess Plateau of China. Trees 21: 357-370.

Zinn KE, Tunc-Ozdemir M, Harper JF. 2010. Temperature stress and plant sexual reproduction: uncovering the weakest links. Journal of Experimental Botany 61: 1959-1968.

Curriculum vitae

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Education

2006	MSc in Bioscience Engineering, Land & Forest Management Faculty of Bioscience Engineering, Ghent University
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Personal experience

2010-2014	PhD research at Ghent University Faculty of Bioscience Engineering Department of Forest and Water Management Financed by BOF
2008-2010	Scientific staff Faculty of Bioscience Engineering Department of Forest and Water Management Financed by Agency for Nature and Forest
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Publications

Publications in international peer reviewed journals

Published or in press

Verheyen K, Adriaenssens S, Gruwez R, Michalczyk I, Ward L, Rosseel Y, Van den Broeck A, García D. 2009. *Juniperus communis*: victim of the combined action of climate warming and nitrogen deposition? Plant Biology 11: 49–59.

De Frenne P, De Schrijver A, Graae BJ, Gruwez R, Tack W, Vandelook F, Hermy M, Verheyen K. 2010. The use of open-top chambers in forests for evaluating warming effects on herbaceous understorey plants. Ecological Research 25: 163–171.

De Frenne P, Kolb A, Graae BJ, Decocq G, Baltora S, De Schrijver A, Brunet J, Chaberie O, Cousins SAO, Dhondt R,Diekmann M, Gruwez R, Heinken T, Hermy M, Liira J, Saquez R, Shevtsova A, Baskin CC, Verheyen K. 2011. A latitudinal gradient in seed nutrients of the forest herb Anemone nemorosa. Plant Biology 13: 493–501.

Tack W, Madder M, De Frenne P, Vanhellemont M, Gruwez R, Verheyen K. 2011. The effects of sampling method and vegetation type on the estimated abundance of *Ixodes ricinus* ticks in forests. Experimental and Applied Acarology: 54: 285–292.

Vanden Broeck A, Gruwez R, Cox K, Adriaenssens S, Michalczyk IM, Verheyen K. 2011. Genetic structure and seed-mediated dispersal rates of an endangered shrub in a fragmented landscape: a case study for *Juniperus communis* in northwestern Europe. BMC Genetics, 12.

De Frenne P, Graae BJ, Kolb A, Shevtsova A, Baeten L, Brunet J, Chabrerie O, Cousins SAO, Decocq G, Dhondt R, Diekmann M, Gruwez R, Heinken T, Hermy M, Öster M, Saguez R, Stanton S, Tack W, Vanhellemont M, Verheyen K. 2011. An intraspecific application of the leaf-height-seed ecology strategy scheme to forest herbs along a latitudinal gradient. Ecography 34: 132–140.

De Frenne P, Brunet J, Shevtsova A, Kolb A, Graae BJ, Chabrerie O, Cousins SAO, Decocq G, De Schrijver A, Diekmann M, Gruwez R, Heinken T, Hermy M, Nilsson C, Stanton S, Tack W, Willaert J, Verheyen K. 2011. Temperature effects on forest herbs assessed by warming and transplant experiments along a latitudinal gradient. Global Change Biology 17: 3240–3253.

Tack W, Madder M, Baeten L, Vanhellemont M, Gruwez R, Verheyen K. 2012. Local habitat and landscape affect *Ixodes ricinus* tick abundances in forests on poor, sandy soils. Forest Ecology and Management 265: 30–36.

De Frenne P, Graae BJ, Rodríguez-Sánchez F, Kolb A, Chabrerie O, Decocq G, De Kort H, De Schrijver A, Diekmann M, Eriksson O, Gruwez R, Hermy M, Lenoir J, Plue J, Coomes DA, Verheyen K. 2013. Latitudinal gradients as natural laboratories to infer species' responses to temperature. Journal of Ecology 101: 784–795.

Gruwez R, Leroux O, De Frenne P, Tack W, Viane R, Verheyen K. 2013. Critical phases in the seed development of common juniper (*Juniperus communis*). Plant Biology 15: 210–219.

De Frenne P, Coomes DA, De Schrijver A, Staelens J, Alexander JM, Bernhardt-Römermann M, Brunet J, Chabrerie O, Chiarucci A, den Ouden J, Eckstein RL, Graae BJ, Gruwez R, Hédl R, Hermy M, Kolb A, Marell A, Mullender SM, Olsen SL, Orczewska A, Peterken G, Petrik P, Plue J, Simonson WD, Tomescu CV, Vangansbeke P, Verstraeten G, Vesterdal L, Wulf M, Verheyen K. 2014. Plant movements and climate warming: intraspecific variation in growth responses to nonlocal soils. New Phytologist 202:431-41.

Gruwez R, De Frenne P, De Schijver A, Leroux O, Vangansbeke P, Verheyen K. 2014. Negative effects of temperature and atmospheric depositions on the seed viability of common juniper (*Juniperus communis*). Annals of Botany 113: 489–500.

Maes S, De Frenne P, Brunet J, de la Peña E, Chabrerie O, Cousins SAO, Decocq, G, Diekmann M, Gruwez R, Hermy M, Kolb A, Verheyen K. 2014. Effects of enhanced nitrogen inputs and climate warming on a forest understorey plant assessed by transplant experiments along a latitudinal gradient. Plant Ecology, in press.

Submitted

Vangansbeke P, Osselaere J, Van Dael M, De Frenne P, Gruwez R, Pelkmans L, Gorissen L, Verheyen K. Sustainability analysis of logging operations in pine stands with additional harvest of woody biomass. Biomass & Bioenergy, submitted.

Gruwez R, Hommel PWFM, De Frenne P, De Schrijver A, Huiskes HPJ, de Waal RW, Vangansbeke P, Verheyen K. Effects of management actions on the recruitment of threatened common juniper populations (*Juniperus communis*). Plant Ecology and Evolution, submitted.

Gruwez R, De Frenne P, De Schrijver A, Vangansbeke P, Verheyen K. Climate warming and atmospheric deposition affect seed viability of common juniper (*Juniperus communis*) via their impact on the nutrient status of the plant. Ecological Research, accepted with major revisions.

In preparation

Gruwez R, De Frenne P, Vander Mijnsbrugge K, Vangansbeke P, Verheyen K. Juniper threatened by climate warming: evidence from warming and transplant experiments along a latitudinal gradient.

Publications in national peer reviewed journals

Gruwez R, Vanden Broeck A, De Frenne P, Tack W, Verheyen K. 2010. Welke toekomst voor de jeneverbes in Vlaanderen: een evaluatie aan de hand van beheerscenario's. Natuur.Focus 9: 167–173.

Vanden Broeck A, Gruwez R, Verheyen K. 2010. De ene jeneverbes is de andere niet: integratie van genetische variatie in een soortbeschermingplan. Bosrevue 33: 9–13.

De Frenne P, Gruwez R, Caron MM, De Schrijver A, Demey A, Hermy M, Verheyen K. 2014. Climate change and Belgian forests: an evaluation of experiments and long-term changes. Natuur.Focus, in press.

Scientific activities

Participation in congresses, symposia or workshops

Participation with oral presentation

Gruwez R, Verheyen K. Status of *Juniperus communis* in Flanders and actions needed for its long-term survival. 6th European conference on ecological restoration (SER). (Ghent, Belgium; September 8 – 12, 2008).

Gruwez R, Verheyen K. Crucial phases in the failing seed production of declining juniper populations. 41st Annual Conference of the Ecological Society of Germany, Switzerland and Austria (GFÖ) (Oldenburg, Germany; September, 5 – 9, 2011).

Gruwez R. Negative effects of temperature and changing precipitation on the seed viability of *Juniperus communis*. International conference on climate change and tree responses in Central European forests (ClimTree). (Zurich, Switzerland; September, 1 – 5, 2013).

Participation with poster presentation

Gruwez R, Leroux O, Verheyen K. Verschillen in de ontwikkeling van het zaad van jeneverbes (*Juniperus communis*) bij populaties met en zonder verjonging. Starters in het bos onderzoek. (Brussels, Belgium; March, 19, 2009).