Comparison of the crop species *Brassica napus* and wild *B. rapa*: characteristics relevant for building up a persistent seed bank in the soil

Tom J. de Jong*, Maria Tudela Isanta and Elze Hesse

Institute of Biology, Leiden University, PO Box 9505, 2300 RA Leiden, The Netherlands

Abstract

Can seed characters be used for predicting the presence of a persistent seed bank in the field? We address this question using ten cultivars of the crop *Brassica napus*, ten feral *B. napus* accessions originating from seeds collected in the field and nine accessions of the closely related ruderal species *Brassica rapa*. When buried for a year in the field, seeds of the wild *B. rapa* displayed, as expected, much higher survival fractions than those of domesticated *B. napus* at two different locations in The Netherlands. Compared to *B. napus*, *B. rapa* produces relatively small seeds with high levels of aliphatic glucosinolates and a thick seed coat. However, within each species none of these characters correlated with seed survival in the soil. At low temperatures, *B. rapa* seeds had lower and more variable germination fractions than those of *B. napus*; a small fraction (4.6%) of the *B. rapa* seeds showed primary dormancy. Rather surprisingly, *B. napus* displayed genetic differences in germination at low temperature, and germination fractions at 5°C correlated negatively with seed survival in the soil. Our comparisons between and within the two species suggest that foregoing germination at low temperatures is an important character for developing a persistent seed bank. We discuss our results in light of environmental risk assessment of genetically modified *B. napus*.

*Correspondence
Fax: + 31-71-5274900
Email: t.j.de.jong@biology.leidenuniv.nl

Introduction

Many species accumulate seeds in the soil. These seeds may survive for many years and germinate after a disturbance to form a new cohort of plants, thereby ensuring population persistence. The seeds of short-lived plants, among which many are weeds, often have the ability to bridge unsuitable periods and to germinate rapidly when disturbance creates a window in time suitable for recruitment (Harper, 1977; Thompson *et al.*, 1998; Baskin and Baskin, 2001). In some species, for instance *Papaver rhoeas*, persistence in the seed bank is clearly the result of seed dormancy. The germination tendency of *Papaver* seeds cycles, as has been shown by taking seeds from the field to a suitable germination environment in the lab at different times of the year (Cirujeda *et al.*, 2006). Other species require light for germination and seeds buried in the soil do not germinate. For a rather large group of species, however, it is mostly unknown which characters enable seeds to persist in the soil. Ripe seeds of *Brassica* species typically germinate 100% when placed under moist and warm conditions, even in complete darkness (Naeem *et al.*, 2009; but see Landbo and Jørgensen, 1997). Yet many species in the Brassicaceae possess a long-term persistent seed bank (Tamis *et al.*, 2003) as is evident from the frequent occurrence of species like *B. rapa*, *B. nigra* and *Sinapis arvensis* along disturbed roadsides and on construction sites throughout western Europe, as well as from the occurrence of volunteers in *B. napus* crops. Without primary dormancy, what characters predispose species or genotypes to build up a long-term persistent seed bank in the soil?

This question is highly relevant for the environmental risk assessment (ERA) of genetically modified (GM) plants (EFSA, 2010). An ERA typically requires data on seed germination for a GM cultivar and its non-GM counterparts. Applicants need to argue whether a new GM cultivar is more or less likely to build up a long-term persistent seed bank in the soil.

Building up a persistent seed bank in the soil requires two things. First, seeds should forgo immediate germination. Mature non-dormant seeds may experience secondary dormancy after seed dispersal; that is, due to exposure to some environmental cue an initially non-dormant seed no longer germinates under conditions that were, at first, conducive for germination. Baskin and Baskin (2001) commented that they were unaware of any species whose initially non-dormant seeds were induced into dormancy. Adler *et al.* (1993) found that 4 weeks of cold stratification decreased subsequent germination of wild *B. rapa* seeds but not that of *B. napus* cultivars. This observation is in line with the assertion of Baskin and Baskin (2001) provided that the wild seeds have some primary dormancy and *B. napus* has not. Several authors (for instance, Pekrun *et al.*, 1997, Gruber *et al.*, 2004; Schatzki *et al.*, 2013) showed that secondary dormancy can be induced in seeds of *B. napus* by keeping them in the dark for 2 weeks in a polyethylene glycol medium that imposes oxygen and water stress (osmotic potential of -0.5 MPa, i.e. permanent wilting point). One can question, however, how often such stressful conditions occur in the agricultural field or in the fertile, disturbed habitats in which feral *B. napus* typically grows. Thus although it is certain that secondary dormancy can be induced under extreme conditions in seeds of *B. napus*, it is not known how often seeds are exposed to such conditions in the field and how efficient this mechanism is in preventing rapid germination. In our view, the role of secondary dormancy in soil seed-bank dynamics should not be taken for granted.

Delayed germination could also simply be due to suboptimal germination conditions. As pointed out by Thompson *et al.* (2003) this should not be considered as dormancy, because seeds readily germinate when exposed to optimal conditions. Hence, the term dormancy is best reserved for those cases where seeds do not germinate upon transfer to optimal conditions. A higher temperature requirement for germination can also have the effect that fewer seeds germinate and remain in the seed bank.

Second, non-germinating seeds should be able to survive in the soil and resist the slow breakdown of the seed coat by micro-organisms and predation by micro-arthropods and earthworms. Gardarin *et al.* (2010) buried seeds of 13 weed species at a depth of 30 cm in the soil and examined survival over 2 years. Survival was positively correlated with thickness of the seed coat, and this character explained 52% of the variation in seed survival. Hendry *et al.* (1994) compared 80 species of the British flora and found a positive correlation between the concentration of phenols and seed survival. They emphasized that phenols defend against microbes and predation, especially under relatively moist conditions. In a review of the subject,

Wagner and Mitschunas (2008) concluded that fungicide treatment typically increases seed survival in the soil. Effects of glucosinolates, i.e. defence compounds mainly found in the Brassicaceae, on seed survival in the soil have not been studied. Yet glucosinolates could well have an effect. Concentrations in seeds are much higher than in the rest of the plant (Clossais-Besnard and Larher, 1991) and *Brassica* crops are being used for biofumigation, which greatly reduces populations of soil arthropods and fungi (Kirkegard *et al.*, 2000).

In this study we compare the seed characteristics of 20 accessions of the crop oilseed rape (*B. napus*) with nine accessions of wild *B. rapa*. The 20 accessions of *B. napus* include modern cultivars, old cultivars and feral populations. We address the following questions. (1) In which aspects does the crop *B. napus* differ from the closely related wild species *B. rapa*? We expect that seed banks are more prevalent in the wild species because domestication selects for 100% germination. The same question applies when comparing different *B. napus* accessions: in feral populations selection may have favoured genotypes with a seed bank (Adler *et al.*, 1993). (2) Which seed characters are correlated with seed survival in the soil? We buried fresh seeds of all 29 accessions at depth 10 cm and measured survival after 1 year in two habitats. The measured characters included thickness of the seed coat, the content of aliphatic and indole glucosinolates in the seed, seed size and germination percentage at low temperatures (5–15°C). (3) Using a subset of accessions, we then ask whether seeds exhibit primary dormancy and whether exposure to the typical field conditions that seeds experience after ripening increases seed dormancy. (4) Finally, we ask whether *B. rapa* and *B. napus* differ in their germination percentage and rate of germination at a temperature range from 15 to 25°C. We expect that for the wild *B. rapa* more seeds enter the seed bank than for the crop *B. napus* and, if this is related to temperature, fewer seeds of *B. rapa* should germinate at cold temperatures than seeds of *B. napus*.

Materials and methods

Species description and collection

B. napus (AACC) is the allotetraploid hybrid of *B. rapa* (AA) and *B. oleracaea* (CC) (U, 1935). Morphologically, *B. rapa* and *B. napus* are very similar but can be distinguished using both flow cytometry and diagnostic characters (Luijten and de Jong, 2010). Originally, cultivars of *B. napus* contained high concentrations of bitter-tasting glucosinolates (GS) and erucic acid. For this reason, modern cultivation mainly uses canola (Gulden *et al.*, 2008), which has been selected to contain very low levels of erucic acid and aliphatic GS

Table 1. Summary of the *Brassica napus* cultivars used and properties of the seeds (glucosinolate content, mass per seed and fraction dry mass in seed coat)

Cultivar	Ref. no.	AGS $\mu M\ g^{-1}$	IGS $\mu M\ g^{-1}$	Mass (mg)	Rel. mass seed coat (%)
Pioneer PR45D03	1B	7.93	0.82	4.85	14.9
Lioness	3A	16.04	2.12	6.85	15.9
Windal*	4A	30.05	2.19	3.47	20.2
Mansholt*	5B	53.09	1.73	3.48	23.1
Velox*	6A	61.21	1.75	4.57	17.3
Billy[†]	7A	9.99	2.42	6.33	16.3
Ladoga[‡]	8B	8.91	1.66	4.70	16.9
Oase[†]	9B	17.30	2.38	3.89	20.2
Hornet[†]	10A	15.19	1.04	4.66	15.1
Pioneer PR46W31	13B	18.95	1.52	3.21	19.1
Average		23.87	1.76	4.60	17.9
CV		0.79	0.31	0.26	0.15

AGS, Aliphatic glucosinolates; IGS, indole glucosinolates; CV, coefficient of variation.
*Old cultivars obtained from the Centre for Genetic Resources in Wageningen.
[†]Cultivars from the German company Eurograss.
[‡]Cultivar from Limagrain.

(especially progoitrin), while retaining indole GS. Large amounts of canola are imported in The Netherlands and other countries of the European Union, and some of the seeds are spilt during transport and/or harvesting of the oilseed crop. *B. napus* seeds may also be introduced in the environment via flower mixtures, birdseed and rodent food. Yet, feral populations of *B. napus* are uncommon in The Netherlands and are mainly located in highly disturbed sites near transhipping locations or canola crops (Luijten and de Jong, 2010). *B. rapa* is a ruderal species commonly found along disturbed roadsides, especially in the west of The Netherlands (Luijten and de Jong, 2010).

In this study we used seeds of 29 different *Brassica* accessions: ten cultivars of *B. napus*, ten feral *B. napus* populations and nine *B. rapa* populations. Seeds of modern cultivars were acquired from breeders (see

Table 1), whereas seeds of old cultivars were obtained from the Centre for Genetic Resources in Wageningen, dating from 1899 (Mansholt's Hamburger), 1959 (Windal) and 1967 (Velox). Seeds of the Utrecht accession (Table 2) were collected from a population near the Cereol factory in Utrecht, which was operational from about 1900 to 2002, and specialized in importing and processing soy in its last decade of activity. It is thus likely that the Utrecht accession originates from a relatively old *B. napus* cultivar. The Almere population was located near a road construction site. Although *B. napus* may have been cropped near Almere in the past, at the time of seed collection no crop was located nearby. All other feral *B. napus* populations from which we collected seeds (Table 2) were located near crops or current transhipping locations. The *B. rapa* accessions (Table 3) were

Table 2. Summary of the feral *Brassica napus* accessions sampled in the field and properties of the seeds

Cultivar/collection*	Ref. no.	AGS $\mu M\ g^{-1}$	IGS $\mu M\ g^{-1}$	Mass (mg)	Rel. mass seed coat (%)
Amsterdam (NH)	2A	31.81	0.96	5.86	11.1
Delfzijl (GR)	2B	11.25	0.76	5.98	16.8
Nieuweschans (GR)	5A	45.88	2.58	5.55	23.2
Almere (FL)	6B	57.06	1.61	4.55	13.6
Nieuwolda (GR)	7B	17.39	1.47	4.26	18.9
Ouddorp (SH)	10B	15.90	1.08	4.78	12.4
Winschoten (GR)	12A	18.96	0.46	3.31	12.1
Utrecht (U)	12B	56.20	1.68	3.99	15.9
Europoort (SH)	14B	17.07	0.63	5.17	14.6
Finsterwolde (GR)	15A	24.67	1.49	4.29	15.7
Average		29.62	1.27	4.77	15.4
CV		0.58	0.49	0.18	0.23

AGS, Aliphatic glucosinolates; IGS, indole glucosinolates; CV, coefficient of variation.
*The province in The Netherlands is given in parentheses: FL, Flevoland; GR, Groningen; NH, North Holland; SH, South Holland; U, Utrecht.

Table 3. Summary of the wild *Brassica rapa* accessions sampled in the wild and properties of the seeds

Cultivar/Collection*	Ref. no.	AGS $\mu M\ g^{-1}$	IGS $\mu M\ g^{-1}$	Mass (mg)	Rel. mass seed coat (%)
Zoelen (GL)*	1A	48.84	0.71	1.47	52.5
Diemen (NH)	3B	60.14	1.34	1.49	29.6
Maarssen (U)	8A	70.39	1.69	1.66	23.3
Breukelen (U)	9A	60.27	1.58	2.05	22.6
Oegstgeest (SH)	11A	64.74	1.32	1.68	29.6
Almere (FL)	11B	51.18	0.42	0.77	33.6
Leiden (SH)	13A	59.43	1.36	0.97	23.8
Montfoort (U)	14A	39.41	0.52	1.51	23.7
Woerden (U)	15B	81.30	1.14	1.02	17.4
Average		59.52	1.12	1.40	28.5
CV		0.20	0.41	0.29	35.9

AGS, Aliphatic glucosinolates; IGS, indole glucosinolates; CV, coefficient of variation.
*The province in The Netherlands is given in parentheses: FL, Flevopolder; GL, Gelderland; NH, North Holland; SH, South Holland; U, Utrecht.

collected along roadsides across The Netherlands in the summer of 2009. All accessions were first grown for one generation in a common garden in Boskoop, starting in September 2009. In the garden, plants of each accession were grown in eight replicate $1 \times 1\ m$ plots that were placed in blocks. A buffer zone of 4 m separated blocks from one another, thereby minimizing outcrossing between different accessions flowering in 2010. We observed that the great majority of bee movements occurred within the $1\ m^2$ plots, so it is likely that gene flow between accessions was limited. We checked this assumption by estimating the percentage of hybridization between *B. rapa* and *B. napus*. In particular, leaf material from a total of 130 individuals was analysed for chromosome number using flow cytometry. Although the species are known to hybridize easily (Anderson and de Vicente, 2010), we found only 3 hybrids in the 130 individuals examined, suggesting that gene flow between accessions does occur but is limited. Seeds were harvested in July 2010, dry stored at room temperature. Then in autumn 2011 these 1-year-old seeds were used for measurements of mass and glucosinolates (Tables 1–3) and a subset of eight accessions (1B, 5B, 10A, 13B, 5A, 10B, 12B and 15A in Tables 1 and 2) was used to examine the effect of temperature on germination rate.

We measured the fresh mass of ten seeds in milligrams. Relative mass of the seed coat was determined by soaking ten seeds in water and then splitting them with a scalpel. After drying at 70°C, the mass of embryos and seed coats was determined separately. Percentage seed coat was calculated as: $100 \times$ dry mass seed coat/(dry mass seed coat + dry mass embryo).

Indole and aliphatic glucosinolates (GS) were determined using high performance liquid chromatography (HPLC) by the Ecogenomics group at the University of Nijmegen (Professr N.M. van Dam) with sinigrin as a reference and extraction in 70%

methanol (van Dam *et al.*, 2004). Seeds contained no aromatic GS.

Temperature was manipulated using a thermo-gradient table manufactured by Flohr instruments (http://www.flohr-instruments.com). Thirty seeds were placed on Whatman 597 filter paper under every bell dome, thereby ensuring seeds made close contact with the table. Ten domes (8.5×8.5 cm with an 8-mm ventilation opening on top) were placed at approximately equal distances on the table, which was abundantly supplied with water throughout the experiment. Temperature was checked with a Eurolec thermometer that was adjusted with a flat metal sensor for improved contact. Over a period of 8 d germinated seeds were counted daily and removed from the table. The eight cultivars were placed simultaneously on the germination table (15–25°C) along with fresh seeds of a control (*B. napus*, collected in July 2011 from a crop grown in Goor, The Netherlands) that was also used in other experiments. This was repeated once, yielding two Petri dishes with in total 60 seeds per line for each temperature. The experiment was repeated in the same way with a gradient from 5 to 15°C. In other experiments reported in this paper we used fresh seeds.

Seed survival in the field

To establish experimental soil seed banks, 50 fresh seeds per accession (Tables 2 and 3) collected at various locations in The Netherlands in the summer of 2009, were mixed with 25 cm³ of local soil and enclosed within nylon mesh bags. For the cultivars (Table 1) we used seeds that were commercially available. Seeds of some cultivars (reference numbers 1B, 3A, 8B, 9B, 10A and 13B, Table 1) had a protective coating against fungal pathogens. The mesh size (0.5 mm²) of the seed bags was small enough for seeds not to get lost through water percolation or soil perturbation, but large enough for the smaller soil fauna to enter.

For each accession, four seed bags were buried at 10 cm depth in September 2009 in two habitats (Boskoop and Swifterband). The fate of seeds was monitored by exhuming the seed bags after 1 year of burial, leading to a maximum number of 200 seeds per population per site at 100% retrieval efficacy. The outside of the seed bags was washed and cleaned of any soil or seeds, after which the content was washed on to sieves of different mesh sizes, thereby removing both coarse and fine soil material, roots and vegetative parts. The number of remaining seeds within each bag after 1 year in the soil was quantified. The viability of these seeds was tested by recording their germination ability after transferring them to moistened filter paper in Petri dishes placed in a growth cabinet providing 16 h light at 24°C. After 30 d the germination test was terminated and the seeds that did not germinate were scarred and placed at 28°C. Just a few seeds germinated at this second treatment, after which all non-germinating seeds were soft and could easily be squeezed. These seeds were clearly dead and decaying. The fraction survival in the field was calculated as the number of seedlings from bags brought to the lab after 1 year that germinated in Petri dishes divided by the number of seeds at the start of the experiment.

Primary dormancy

Before starting this experiment we tested whether darkness affected germination of 20 fresh seeds of six *B. napus* and two *B. rapa* accessions, and found that darkness had no inhibiting effect on % germination (Table 4, paired sample Wilcoxon test, $P = 0.31$). Based on this result, all germination experiments were carried out using fluorescent lighting. Primary dormancy was tested for three accessions: *B. napus* seeds originating from an agricultural field cropped with a cultivar of unknown origin in Goor (The Netherlands); *B. rapa* seeds collected at two different locations in Maarssen

along the River Vecht, The Netherlands, at the same location as mentioned in Table 3. Fresh seeds were collected in July 2011 when fruits were fully ripe (brown), were kept at room temperature and then used for the primary and secondary dormancy experiments that started in October 2011. To see whether freshly collected seeds germinate under optimal conditions, seeds were placed in Petri dishes with abundant water at 28°C and 16 h fluorescent light. This constant temperature was chosen simply because it produced very high germination percentages for both *Brassica* species. In another study on primary germination of *B. rapa* (Lando and Jørgensen, 1997) seeds were first exposed for 4 d at constant 20°C, then 3 d at 16 h 20°C/8 h 30°C and finally 4 d at 16 h 20°C/8 h 30°C with 0.2% KNO_3. These authors emphasized that changing temperatures promoted germination, so our method could have underestimated the % germinating seeds under the most suitable conditions. We tested for viability by scarring the non-germinated seeds and returning them to favourable conditions for germination. All remaining non-germinating seeds were clearly dead at the end of the experiment, which was assessed by squeezing the seeds. For each accession we used three Petri dishes containing 20 seeds each.

Secondary dormancy

We used the same fresh seeds (July 2011) as in the primary dormancy experiment and applied the treatments in October 2011. The following four treatments reflect the natural conditions seeds are exposed to in the soil during the summer to winter transition. After seed ripening in June and July, seeds are exposed to high summer temperatures, they then land on the ground amongst the vegetation. Seeds are gradually buried in the soil, after which they experience winter cold. In the first treatment, seeds were kept dry for 4 weeks at 30°C (heat treatment).

Table 4. Percentage germination* of eight selected cultivars under light and dark conditions at 20°C

Species	Cultivar/accession	Ref. no.	% Germination light	% Germination dark
B. napus	Windal	4A	95.0	100.0
B. napus	Mansholt	5B	60.0	100.0
B. napus	Hornet	10A	88.9	82.4
B. napus	Delfzijl	2B	85.0	83.3
B. napus	Ouddorp	10B	94.7	95.0
B. napus	Utrecht	12B	47.4	94.4
B. rapa	Maarssen	8A	33.3	31.3
B. rapa	Breukelen	9A	5.3	25.0

*Germination of viable seeds, excluding the seeds that turned out to be dead after scarring and a second germination opportunity.

In the second, seeds were kept for 4 weeks under moist conditions under a thick cover of green leaves in the field (shade treatment). In the third, seeds were kept for 4 weeks in complete darkness in the field under moist conditions (dark treatment). In the last treatment, seeds were kept for 4 weeks under moist conditions in a refrigerator (5°C). All seeds that were kept under moist conditions were treated afterwards with a 25% chlorine solution that effectively stopped fungal growth. Control seeds were stored in paper envelopes and kept in the lab for the same period of time. All seeds were then exposed to constant temperature ranging from 15 to 25°C on the thermo-gradient table under fluorescent light. Constant temperatures are the simplest way to quantify and compare germination behaviour of seeds but changing temperatures would also be possible and more natural. In each run we compared nine columns and these always included the three control columns with untreated seeds (*B. napus*, *B. rapa* Maarssen 1 and 2) and the remaining six columns with one specific treatment each. Results on average germination fraction of 30 seeds per dome were statistically analysed.

Comparative germination of *B. napus* and *B. rapa*

Germination of fresh seeds of *B. napus* and *B. rapa* was compared using the controls of the secondary dormancy experiment. However, we did not use the data from the controls belonging to the heat treatment, because germination was low in both treatment and control, probably due to water shortage. In total we pooled data from three temperature columns so that for each temperature, the germination fraction is based on three Petri dishes, yielding 90 seeds in total.

Statistics

We calculated Spearman rank correlations when variables were not normally distributed. In the secondary dormancy experiment we examined effects of treatment (tmt) of the seeds on % germination at different temperatures (T) and for two different accessions (ac). We used the procedure linear model lm (formula: %germ = T*tmt*ac) from the statistical package *R* (http://www.r-project.org/) to examine results, which generates a table of effects of the factors tmt and ac and of the covarariate T on the dependent variable % germination and of all interactions. When the interactions were not significant we ran the model again without the interactions and present the latter output. All residuals of these models fitted a normal distribution. Similarly, in the species comparison paragraph we tested for effect of ac and T on % germination in the linear model %germ = T*ac.

Results

Comparison of seed characters between species and accessions

Seeds of the old cultivars Windal, Mansholt and Velox typically had high levels of aliphatic GS (ranging from 30.05 to 61.21 μM g^{-1}) while the new cultivars had lower levels and a smaller range (between 7.93 and 18.95 μM g^{-1}, Table 1). This result was expected given the domestication history. There were no consistent differences between old and new cultivars in either indole GS or morphological seed characters. Five out of the ten feral *B. napus* accessions had higher aliphatic GS concentrations than any of the new cultivars (Table 2), suggesting they might be derived from old cultivars. Morphologically, seeds of feral *B. napus* are similar to those of cultivated *B. napus*. *B. rapa* had high concentrations of aliphatic GS (between 39.41 and 81.30 μM g^{-1}, Table 3). The average seed mass of the *B. rapa* accessions was less (1.40 mg) than that of cultivated (4.60 mg) or feral (4.77 mg) *B. napus* accessions. Seeds of *B. rapa* had a relatively thicker seed coat. All these differences were highly significant in a Kruskal–Wallis non-parametric one-way ANOVA (all *P* values < 0.01), and can account for between-species differences. The level of indole GS differed significantly between the three groups (Kruskal–Wallis *P* = 0.025), being marginally lower in *B. rapa*.

Seed characters that allow survival in the soil

Because seed survival was not normally distributed, data were analysed using non-parametric tests (medians given below). Seed survival was much higher in the dry habitat compared to the wet habitat (cultivated *B. napus* = 0.29 and 0.005; feral *B. napus* = 0.11 and 0.085; wild *B. rapa* = 0.60 and 0.45 in Swifterbant and Boskoop, respectively). Differences between the three groups were significant in both habitats (Kruskal–Wallis test: *P* = 0.0088 and *P* = 0.005 in Swifterbant and Boskoop, respectively), with seed survival being high for *B. rapa* and low for both the cultivated and feral *B. napus* lines.

If we take all 29 cultivars and accessions of the two species together, there is a positive correlation in seed survival in Boskoop and in Swifterbant (Spearman rank correlation: τ = 0.44, *P* = 0.017, *n* = 29). However, this mainly reflects that *B. rapa* seeds survive better than *B. napus* seeds in both habitats. When analysing each species on its own, we found no correlation between survival in Boskoop and survival in Swifterband (τ = 0.19, *P* = 0.62 for the 9 *B. rapa* accessions and τ = 0.00, *P* = 0.99 for the 20 *B. napus* cultivars and accessions).

Using the whole dataset (*n* = 29), survival in Swifterbant correlated significantly with aliphatic GS
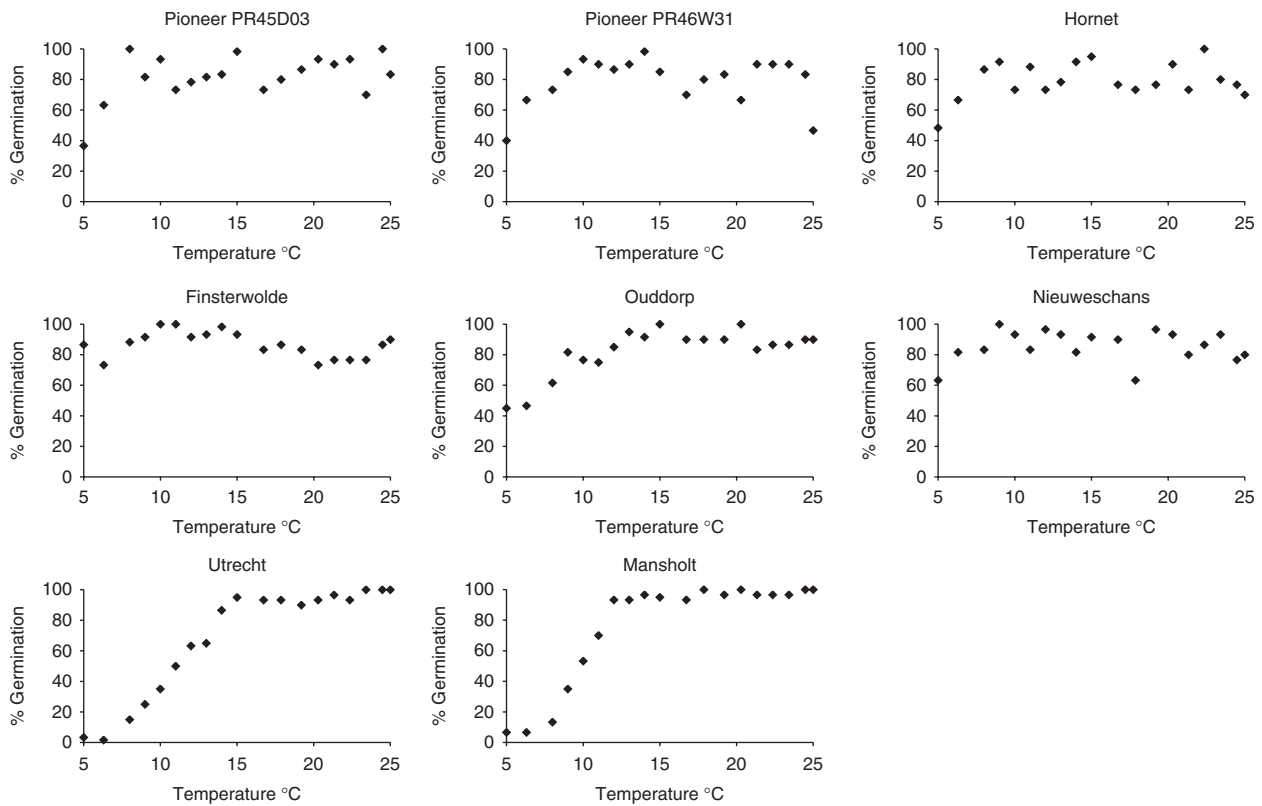
Figure 1. Temperature-dependent germination of 1-year-old seeds of various *Brassica napus* accessions: top, three modern cultivars; middle, three feral accessions; bottom, feral accession from Utrecht and the old crop Mansholt's Hamburger.

content ($\tau = 0.46$; two-sided $P = 0.01$), fractional seed coat biomass ($\tau = 0.49$; $P = 0.07$) and seed mass ($\tau = -0.46$; $P = 0.012$). In Boskoop, survival of seeds correlated significantly with aliphatic GS ($\tau = 0.49$; $P = 0.007$, $n = 29$), fractional seed coat biomass ($\tau = 0.41$; $P = 0.028$) and seed mass ($\tau = -0.75$; $P < 0.001$). All correlations were in the expected direction. However, large between-species differences in all measured characters confound these correlations (i.e. *B. rapa* seeds have higher levels of aliphatic GS, a thicker seed coat and smaller seed mass). When examining *B. napus* and *B. rapa* separately, only two correlations were significant in Boskoop: in *B. napus*, seed mass correlated negatively with survival ($\tau = -0.49$; $P = 0.029$), whereas in *B. rapa* fractional seed coat biomass correlated negatively with survival ($\tau = -0.78$; $P = 0.013$). However, after Bonferroni correction none of these correlations was significant at $\alpha = 0.05$, so they should be discarded.

For a subset of eight *B. napus* accessions, both cultivated and feral, we determined seed germination of 1-year-old seeds at low temperatures. We expect a higher percentage of the seeds to be lost from the soil seed bank when seeds are able to germinate at a wide range of temperatures. While six *B. napus* accessions invariably show high germination percentages at temperatures of 5°C or higher, two accessions show a strong decline in germination percentage when temperatures drop below 13°C (Fig. 1): feral *B. napus* from Utrecht and the old cultivar Mansholt both had high seed survival in Swifterbant (63.5 and 50.5%, respectively). The Spearman rank correlation between average germination over 5–15°C and survival in Swifterbant was $\tau = -0.60$ ($P = 0.12$). The rank correlation was significant when solely looking at germination at 5°C ($\tau = -0.83$; $P = 0.01$). There were no significant correlations between germination at 5–15°C and seed survival in Boskoop, which is not surprising given that all *B. napus* seeds survived very poorly in wet Boskoop soil (Fig. 2).

Primary dormancy

Out of the 60 fresh seeds of *B. napus* examined, one was dead and 59 germinated immediately at 28°C. Of the 120 *B. rapa* seeds, 114 germinated immediately. After scarring, five additional seeds germinated while one seed was dead. A small fraction of the *B. rapa* seeds (5/119 = 4.2%) apparently delays germination even when conditions are favourable, and thus has primary dormancy.
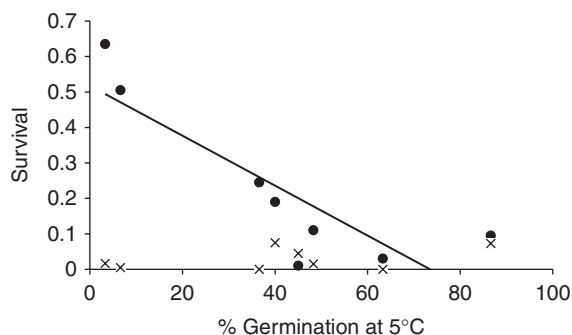
Figure 2. Correlation between germination percentage at low temperature (5°C) of 1-year-old *Brassica napus* seeds and fraction survival of seeds in the soil in a dry habitat (Swifterbant, closed dots) and in a wet habitat (Boskoop, crosses).

Secondary dormancy

Germination of fresh seeds of *B. napus* was not affected by any of the imposed treatments, seeds typically had very high germination rates between 90 and 100%.

In *B. rapa* heat had no significant effect on germination of fresh seeds (results not shown). Dark storage was the only treatment that reduced seed % germination ($P = 0.04$), and the extent of the effect differed between the two *B. rapa* accessions (significant treatment × accession interaction term: $P = 0.04$; Fig. 3, Table 5). In particular, dark storage decreased germination in one accession (average germination Maarssen 1 over whole temperature range: control = 39.3% and dark = 24.3%), but not in the other (average germination Maarssen 2: control = 30.6% and dark storage = 31.1%).

Cold storage strongly increased germination percentage ($P < 0.0001$), irrespective of the accession and germination temperature used (none of the interactions
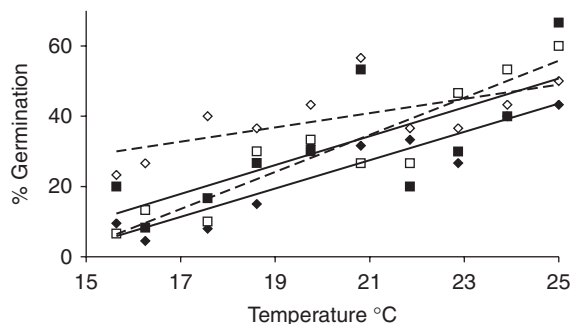

Figure 3. Dark storage for 4 weeks in the field reduced percentage germination of fresh seeds of the *B. rapa* accession Maarssen 1 (open diamonds and broken regression line = control, closed diamonds and solid line = dark), but had no effect on Maarssen 2 (open squares and broken line = control, closed squares and solid regression line = dark).

significant, table not shown). Averaged over all temperatures (15–25°C), cold storage for 4 weeks increased germination from 39.9 to 68.8%. At 25°C only one of the 120 seeds of the two accessions did not germinate.

Covering seeds with leaves similarly increased subsequent germination percentage ($P = 0.002$, table not shown), but the extent depended on the accession (significant treatment × accession interaction term: $P = 0.03$) and germination temperature used (accession × temperature interaction term: $P = 0.01$).

In summary, germination behaviour of *B. rapa* seeds is affected by many factors in a complex way. Dark storage had no effect in one accession but decreased % germination in another accession. Dark storage is the only factor that slightly increased seed dormancy. Cold and covering with leaves both increased germination fractions, which could simply be due to deterioration of the seed coat or leakage of inhibiting hormones from the seed. Germination of *B. napus* seeds was not affected by treatment.

Species comparison

Fresh *B. napus* seeds (collected from a field in Goor) germinated to nearly 100% across the whole temperature gradient (Fig. 4). In contrast, seed germination of fresh *B. rapa* seeds was lower, and differed between the two Maarssen accessions (linear model %germ = ac + t, $P < 0.0001$). Interestingly, between-species differences declined with increased temperature. Days until germination at 20°C was typically 1.4 d for *B. napus*, and 1.9 or 2.4 d for *B. rapa* (Maarssen 1 and 2, respectively). Low temperature slowed down germination, in particular in *B. rapa*. The data were fitted by linear regression for each of the three lines (see Table 6).

Discussion

Species comparison

We found wild *B. rapa* to differ markedly from the closely related crop species *B. napus*: seeds of *B. rapa* were smaller, had a thicker seed coat, a higher content of aliphatic glucosinolates and lower germination percentages at moderate temperatures. In addition, a small fraction (4.2%) of *B. rapa* seeds displayed primary dormancy, whereas *B. napus* seeds all germinated under optimal conditions. The germination fraction of *B. rapa* can be modified by environmental factors, but not that of *B. napus*. Moreover, *B. rapa* seeds had a higher survival fraction in the soil seed bank than those of *B. napus*. These differences are congruent with expectations for wild crop species comparisons and are in agreement with findings of other studies. For instance, Hails *et al.* (1997) found

Table 5. Output of a linear model that predicts % germination (75% explained variance) of *Brassica rapa* from temperature (T), treatment (tmt, control or dark storage) and accession (ac, Maarssen 1 and Maarssen 2) and their interactions

Variable	Coefficient	SE	t
Intercept	− 1.53	18.20	− 0.084*[a]
Temperature	2.02	0.88	2.27*
Accession [Maarssen 2][b]	− 73.95	25.74	− 2.87**
Treatment [dark]	− 55.13	25.74	− 2.14*
Interaction T × ac[Maarssen 2]	3.22	1.25	2.56*
Interaction T × tmt[dark]	1.98	1.25	1.58
Interaction ac[Maarssen 2] × tmt[dark]	78.83	36.40	2.16*
Interaction T × ac[Maarssen 2] × tmt[dark]	− 3.13	1.77	− 1.76

[a] Statistical significance (two-sided): *, $P < 0.05$; **, $P < 0.01$.
[b] Notation means that for accession Maarssen 2 −73.95 needs to be added in the linear equation (see Material and methods) that predicts % germination, while the default value for Maarssen 1 is 0.

1.5% survival of *B. napus* seeds at 10 cm in the soil after 1 year, whereas seeds of the ruderal *Sinapis arvensis* had 60% survival under the same conditions. Adler *et al.* (1993) found that germination of modern domesticated *B. rapa* cultivars was always 100%, whereas wild *B. rapa* accessions had lower germination percentages that were affected by many interacting environmental factors. As Adler *et al.* (1993) argued, variable germination responses could aid seeds to germinate at different moments in time, thereby increasing persistence in natural environments.

Other comparisons

Although old cultivars of *B. napus* did not differ with respect to their seed morphology, they did contain high levels of aliphatic GS compared to modern cultivars. Interestingly, we found evidence for genetic variation in *B. napus* with respect to germination at low temperature; the old accessions Mansholt's Hamburger (1899) and Utrecht (feral) displayed low or no germination at 5°C. Consistently, Marshall and Squire (1996) found, at 4.8°C, relatively high germination percentages in a new double zero line (Rocket; *c.* 90%) compared to an old line (Martina; 60%).

Seeds collected in feral populations of *B. napus* did not differ from cultivars with respect to either their morphological characters or temperature-dependent germination response. This suggests that rapid natural selection for weedy characters that allow the formation of a long-term persistent seed bank is lacking.

Dark dormancy

In the Swifterbant habitat, considerable fractions of the *B. rapa* seeds (between 13.5 and 79.5%) and *B. napus*

seeds (between 0 an 63.5%) survived 1 year in the soil. Primary dormancy was so low in our accessions that it cannot explain seeds not germinating during a year in the soil. Our results indicate that foregoing germination at low temperatures increases seed survival. However, temperatures in June–September at 10 cm depth typically vary between 15.3 and 18.1°C (www.knmi.nl), falling well within the temperature range at which all the examined *B. napus* lines germinated 100%, and it is therefore likely that a single rain shower is enough to induce germination.

Alternatively, seeds may develop dark dormancy when buried in the soil, i.e. seeds lose the ability to germinate even under otherwise optimal soil conditions if they remain in the dark (Pons, 1991). When exposed to a single light flash, seeds germinate again, so that it is essential to keep seeds always in the dark in such experiments. Instead, in this paper we always tested seeds under fluorescent light after treatment for 4 weeks in darkness. Pekrun *et al.* (1998) exposed *B. napus* seeds to a 0–4-week period of darkness and
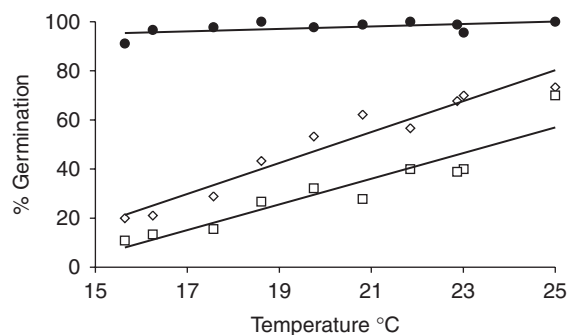

Figure 4. Comparison of temperature-dependent germination of fresh seeds of *Brassica napus* (closed dots) and two *Brassica rapa* accessions collected near Maarssen (1, open diamonds; 2, open squares).

Table 6. Linear regression of days until germination (*D*) as a function of temperature (*T* in °C)

	D at 20°C	Intercept	95%-CL	Slope	95%-CL	*r*
B. napus	1.42	2.49	2.10/2.87	−0.053	−0.072/−0.034	0.91
B. rapa 1	1.95	3.89	2.72/5.06	−0.097	−0.154/−0.039	0.81
B. rapa 2	2.41	6.80	4.19/9.40	−0.218	−0.347/−0.091	0.81

CL, confidence limits.

then tested germination in the dark. They found that the percentage of non-germinating seeds increased from 1.6% (no dark storage) to 37.2% (4 weeks in darkness). Studies on the effect of management on seed bank persistence and volunteer emergence found that without tillage the seed bank of *B. napus* is essentially zero (Pekrun *et al.*, 1998; Lutman *et al.*, 2003, 2005), indicating that seeds cannot develop secondary dormancy when dispersed on top of the soil. *B. napus* seeds are known to survive for up to a period of 10 years when foregoing germination (D'Hertefeldt *et al.*, 2008). Although these findings suggest that dark dormancy could be important for the soil seed bank, several aspects require further investigation. Buried seeds may need several weeks to acquire dark dormancy (Pekrun *et al.*, 1998) and during that period they could easily germinate. Not germinating at low temperature, as we found for the Utrecht and Mansholt accessions, may allow more seeds to acquire dark dormancy. In a recent paper, Schatzki *et al.* (2013) demonstrated genetic variation in the ability of *B. napus* cultivars to develop dark dormancy in polyethylene glycol, under darkness and osmotic stress. A mutant without secondary dormancy would potentially solve the seed bank problem for GM canola and the problem of emerging volunteers in canola cropping.

Evaluation of seed banks in environmental risk assessment

EFSA (2010) guidance requires applicants to specify in the ERA persistence and invasiveness of a crop species. Developing a long-lasting seed bank is a weedy character and information on this is relevant for the ERA. Besides observations on the occurrence of volunteers in subsequent crops, EFSA (2010, p. 45) suggests including information on seed germination and seed burial experiments. In current ERAs applicants typically perform germination experiments at room temperature and with light. However, when all seeds germinate (no primary dormancy) this is no guarantee that the species in question cannot develop a persistent soil seed bank in the field. Testing fresh seeds in the dark makes no difference for *B. napus* because they will rapidly germinate. An interesting option would be to induce secondary dormancy in the seeds using the polyethylene glycol methods of

Schatzki *et al.* (2013) and others. It seems reasonable to expect that the fewer seeds can be induced into secondary dormancy, the smaller the persistence in the soil seed bank. Gruber *et al.* (2004) validated the polyethylene glycol method, showing a positive correlation (0.61–0.80) between the fraction of seeds induced into secondary dormancy and seed survival in burial experiments in the field. In addition, we suggest that it could also be informative to perform germination tests at cold and cool temperatures. The ability not to germinate at low temperatures could also be a proxy for developing a persistent seed bank for *B. napus*. The longer the period of foregoing germination, the higher the chance a seed can acquire secondary dormancy and then persist for a very long period in the dark in the soil.

We found that the cultivars and accessions for which the seeds survived best in Boskoop were not the ones that survived best in Swifterbant. A problem with using the seed burial experiments in the ERA may therefore be that results for one habitat cannot easily be extrapolated to another.

References

Adler, L.D., **Wikler, K.**, **Wyndham, F.S.**, **Kinder, C.R. and Schmitt, J.** (1993) Potential for persistence of genes escaped from canola: germination cues in crop, wild, and crop-wild hybrid *Brassica rapa*. *Functional Ecology* **7**, 736–745.

Anderson, M.S. and de Vicente, M.C. (2010) *Gene flow between crops and their wild relatives*. Baltimore MA, Johns Hopkins.

Baskin, C.C. and Baskin, J.M. (2001) *Seeds; ecology, biogeography, and evolution of dormancy and germination*. San Diego, Academic Press.

Cirujeda, A., **Recasens, J. and Taberner, A.** (2006) Dormancy cycle and viability of buried seeds of *Papaver rhoeas*. *Weed Research* **46**, 327–334.

Clossais-Besnard, H. and Larher, F. (1991) Physiological role of glucosinolates in *Brassica napus*; concentration and distribution pattern of glucosinolates among plant

organs during a complete life cycle. *Journal of Science Food and Agriculture* **56**, 25–38.

D'Hertefeldt, T., Jørgensen, R.B. and Pettersson, L.B. (2008) Long-term persistence of GM oilseed rape in the seedbank. *Biology Letters* **4**, 314–317.

EFSA (2010) Guidance on the environmental risk assessment of genetically modified plants. *EFSA Journal* **8**, 1879.

Gardarin, A., Durr, C., Mannino, M.R., Busset, H. and Colbach, N. (2010) Seed mortality in the soil is related to seed coat thickness. *Seed Science Research* **20**, 243–256.

Gruber, S., Pekrun, C. and Claupein, W. (2004) Seed persistence of oilseed rape (*Brassica napus*), variation in transgenic and conventionally bred cultivars. *Journal of Agricultural Science* **142**, 29–40.

Gulden, R.H., Warwick, S.I. and Thomas, A.G. (2008) The biology of Canadian weeds. 137. *Brassica napus* L. and *B. rapa* L. *Canadian Journal of Plant Science* **88**, 951–996.

Hails, R.S., Rees, M., Kohn, D.D. and Crawley, M.J. (1997) Burial and seed survival in *Brassica napus* subsp. *oleifera* and *Sinapis arvensis*; a comparison of transgenic and non-transgenic lines of the crop. *Proceedings of the Royal Society B Biological Sciences* **264**, 1–7.

Harper, J.L. (1977) *Population biology of plants*. London, Academic Press.

Hendry, G.A.F., Thompson, K., Edwards, E. and Thorpe, P.C. (1994) Seed persistence, a correlation between seed longevity and ortho-dihydroxyphenol concentration. *Functional Ecology* **8**, 658–664.

Kirkegard, J.A., Sarwar, M., Wong, P.T.W., Mead, A., Howe, G. and Newell, M. (2000) Field studies on the biofumigation of take-all by *Brassica* crops. *Australian Journal of Agricultural Research* **51**, 445–456.

Landbo, L. and Jørgensen, J.B. (1997) Seed germination in weedy *Brassica campestris* and its hybrids with *B. napus*: implications for risk assessment of transgenic oilseed rape. *Euphytica* **97**, 209–216.

Luijten S.H. and de Jong T.J. (2010) A baseline study of the distribution and morphology of *Brassica napus* L. and *Brassica rapa* L. in the Netherlands COGEM Report 2010-03. Available at www.cogem.net (accessed 17 May 2013).

Lutman, P.J.W., Freeman, S.E. and Pekrun, C. (2003) The long-term persistence of seeds of oilseed rape (*Brassica napus*) in arable fields. *Journal of Agricultural Science* **141**, 231–240.

Lutman, P.J.W., Berry, K., Payne, R.W., Simpson, E., Sweet, J.B., Champion, G.T., May, M.J., Wightman, P., Walker, K. and Lainsbury, M. (2005) Persistence of seed from crops of conventional and herbicide tolerant oilseed rape (*Brassica napus*). *Proceedings of the Royal Society B Biological Sciences* **272**, 1909–1915.

Marshall, B. and Squire, G.R. (1996) Non-linearity in rate–temperature relations of germination in oilseed rape. *Journal of Experimental Botany* **47**, 1369–1375.

Naeem, M.S., Liu, D., Raziuddin, R., Wan, G.L., Tang, G.X. and Zhou, W.J. (2009) Seed dormancy and viability. pp. 151–176 in Gupta, S.K. (Ed.) *Biology and breeding of crucifers*. Boca Raton, CRC Press.

Pekrun, C., Lutman, P.J.W. and Baeumer, K. (1997) Induction of secondary dormancy in rape seeds (*Brassica napus* L.) by prolonged imbibition of water stress or oxygen deficiency in darkness. *European Journal of Agronomy* **6**, 245–255.

Pekrun, C., Hewitt, J.D.J. and Lutman, P.J.W. (1998) Cultural control of volunteer oilseed rape (*Brassica napus*). *Journal of Agricultural Science* **130**, 155–163.

Pons, T.L. (1991) Induction of dark dormancy in seeds: its importance for the seed bank in the soil. *Functional Ecology* **5**, 669–675.

Schatzki, J., Allam, M., Klöppel, C., Nagel, M., Börner, A. and Möllers, C. (2013) Genetic variation for secondary seed dormancy and seed longevity in a set of black-seeded European winter oilseed rape cultivars. *Plant Breeding* **132**, 174–179.

Tamis W.L.M., van der Meijden, R., Runhaar, J., Bekker, R.M., Ozinga, W.A., Ode, B. and Hoste, I. (2003) Standaardlijst Nederlandse Flora 2003. Available at http://www.floron.nl (accessed 17 May 2013).

Thompson, K., Bakker, J.P., Bekker, R.M. and Hodgson, J.P. (1998) Ecological correlates of seed persistence in soil in the north-west European flora. *Journal of Ecology* **86**, 163–169.

Thompson, K., Cerlani, R.M., Bakker, J.P. and Bekker, R.M. (2003) Are seed dormancy and persistence in soil related? *Seed Science Research* **13**, 97–100.

U, N. (1935) Genome analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. *Japanese Journal of Botany* **7**, 389–452.

van Dam, N.M., Witjes, L. and Svatos, A. (2004) Interactions between aboveground and belowground induction of glucosinolates in two wild *Brassica* species. *New Phytologist* **161**, 801–810.

Wagner, M. and Mitschunas, N. (2008) Fungal effects on seed bank persistence and potential applications in weed biocontrol: a review. *Basic and Applied Ecology* **9**, 191–203.