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Seed viability of common ragweed (*Ambrosia artemisiifolia* L.) is affected by seed origin and age, but also by testing method and laboratory

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Abstract

Common ragweed (*Ambrosia artemisiifolia* L.) is an annual *Asteraceae* species native to North America which is highly invasive across Europe and has harmful impacts, especially on human health and agricul-

ture. Besides its wide ecological range, particularly its high reproductive power by seeds is promoting its spread to various habitats and regions. To prevent further spread and to control the plant, the European Commission funded projects and COST-Actions involving scientists from all over Europe. A joint trial was set up comprising eight different laboratories from Europe to study seed viability variation in different seed samples. Three different testing methods (viability test with 2,3,5-triphenyltetrazolium chloride (TTC), a germination test combined with a subsequent TTC test and a crush test) were tested within the EU-COST-Action SMARTER network to four different seed origins. The viability test results from different laboratories were compared for variation amongst tests and laboratories. The main aim was to optimise the reliability of testing procedures, but results revealed not only significant effects of seed origin and seed age on seed viability, but also considerable differences between the output of the individual testing methods and furthermore between laboratories.

Due to these significant differences in the results of the testing labs, additionally a second test was set up. Twelve Austrian ragweed populations were used for TTC testing to obtain a precise adjustment of the testing method as well as a tight guideline for interpreting the results, particularly for the TTC state "intermediate" since a proper classification of TTC-intermediate coloured seeds is still a challenge when determining viability rates.

Keywords

Common ragweed, germination rate, seed age, seed origin, 2,3,5-triphenyltetrazolium chloride (TTC), viability testing, crush test

Introduction

Information on seed viability is of significant importance, not only in agricultural science, but also in the field of invasion ecology. Beyond fecundity and dispersal capacities, the fate of seeds of invasive alien species (IAS) after arrival to a new site is essential in determining the outcome of invasion (Moravcová et al. 2006; Fumanal et al. 2008). Especially for annual invasive alien species, production and performance of seeds is the main driver of naturalisation (Richardson et al. 2000). In this context, seed persistence is often associated with invasion success, since the ability of delaying seed germination through time is a bet-hedging strategy that spreads the risk of reproductive failure. This is essential in unpredictable, newly-conquered environments where the risk of dying before reaching maturity is high (Venable and Brown 1988; Ooi 2012; Gioria et al. 2012; Long et al. 2015). Thus, most of the IAS are ruderals that are well adapted to disturbances due to their long-lived seed banks (Grime 2001; Fumanal et al. 2008). One of the economically most important IAS in Europe is common ragweed (*Ambrosia artemisiifolia* L.), an annual *Asteraceae* species native to North America which is not only one of the most dominant inducers of pollen allergy, but also a troublesome agronomic weed (Fumanal et al. 2007; Bullock 2010; Smith et al. 2013; Schaffner et al. 2020). Extensive populations of common ragweed are known in Austria, Croatia, France, Germany Hungary, Italy, Romania, Russia, Serbia, Slovenia, Switzerland, (Northern) Turkey and Ukraine (Kazinczi et al. 2008; Essl et al. 2015; Ozaslan et al. 2016; Zambak and Uludağ 2019). Its large ecological amplitude enables the species to be a successful pio-

neer in early successional stages and in several habitat types (Fumanal et al. 2008a). In agricultural areas, common ragweed is one of the most important weeds in spring crops like sunflower, soybean, maize, sugar beet and oil seed pumpkin. Additionally, it also colonises other human-disturbed habitats, such as roadsides, construction sites, abandoned gravel pits and riverbanks (Fumanal et al. 2008b; Essl et al. 2015,). Last, but not least, common ragweed is a serious threat to human health due to abundant allergenic pollen release. The pollen production varies amongst plants and years from 0.1 to 3.8 billion pollen grains per plant which become airborne immediately when conditions are favourable (Fumanal et al. 2007; Smith et al. 2013; Katz and Batterman 2019). In North America and parts of Europe, the pollen of the species is the main cause of hay fever and allergic rhinitis, causing an estimated financial burden for the health system of approx. 630 Euro per year per each person concerned (Wopfner et al. 2005; Jäger 2006; Ziska et al. 2011). Schaffner et al. (2020) even estimated direct and indirect costs caused by common ragweed in the European Union by Euro 7.4 billion per year.

Particularly, its success as an IAS is tightly associated with its high production of seeds. On average, one plant produces 1,500 to 3,000 seeds. The highest reported number of seeds per plant has been found in Russia with a total number of approx. 62,000 seeds on one single plant (Fisjunov 1984). The dispersal unit of common ragweed is often called "achene". It consists of a durable involucrum covering a hard-coated fruit (= achene s. str); the single seed (morphological term!) inside the achene is soft and comprises of a well-developed embryo. For simplicity, the term "seed" will be used in the subsequent text to describe the dispersal unit of common ragweed. When seeds of common ragweed mature in autumn, they are innately dormant (primary dormancy). Seeds in primary dormancy require moist chilling (cold stratification) to come out of dormancy, which occurs during winter (Payne and Kleinschmidt 1961; Baskin and Baskin 1980). Under laboratory conditions, Baskin and Baskin (1987) recommended a wet and dark stratification at 4 °C for 2 weeks to obtain about 75% of germination. Since Pickett and Baskin (1973) demonstrated higher germination rates with increasing length of stratification, chilling treatments of 6 weeks and more are recommended for maximum germination percentage by other authors (Willemsen and Rice 1972; Leiblein et al. 2014; Onen et al. 2020). However, less is known about storage suitability of common ragweed seeds. In general, the life span of seeds is determined by their genetic and physiological storage potential and by any deteriorating events that occur prior to or during storage, as well as by the interaction with environmental factors (Bewley and Black 1994). Even though Toole and Brown (1946) stated that seeds of common ragweed can remain dormant in the soil seed bank for up to 39 years, it is evident that long-term storage of seeds generally reduces their viability and vigour, even if the seeds are stored *ex situ* under stable conditions (Kazinczi et al. 2008; Long et al. 2015; Starfinger and Karrer 2016).

Seed viability and performance is crucial to understand the ecological niche and expansion of annual weeds, i.e. when weed management systems are to be established (Zimdahl 2018). Seed viability is commonly evaluated by a germination test, colouration test with 2,3,5-triphenyltetrazolium chloride (TTC) and crush test. It is not clear at the present time which test gives most reliable results and which is most easily applicable.

To prevent further spread and to control or eradicate this IAS, the European Commission funded the project "Assessing and controlling the spread and the effects of common ragweed in Europe". Within the framework of this project ("HALT AMBROSIA"), a consortium of scientists from five countries established a viability test by colouration of living ragweed seeds by TTC. The first results indicated that differences in TTC classification of different seed lots by different labs were higher than the variation between the seed origins (Starfinger et al. 2012; Karrer et al. 2016b). Hitherto existing results about seed biology were transferred to many stakeholders via the EU-funded FA 1203 COST-action SMARTER (Sustainable management of *Ambrosia artemisiifolia* in Europe, Müller-Schärer et al. 2018), an interdisciplinary network of more than 120 experts involved in the control of common ragweed in more than 30 countries.

The main goal of this paper is to achieve better insight into germination biology and viability testing as part of monitoring tools against ragweed. Therefore, in 2015 a joint trial was set up within SMARTER, comprising eight different European laboratories (including the five labs from the first joint trial within HALT AMBROSIA), to evaluate three different viability testing methods (colouration of living tissue by TTC, germination test combined with a subsequent TTC test and a crush test). We used four different seed origins (two different sites each, in Austria and in Hungary), aiming at the optimisation of testing procedures on the viability of ragweed seeds. The specific aim of this study was to detect possible differences in the viability status between: 1) the seed origins and ages, 2) the testing labs and 3) the testing methods.

Due to disagreements of the participating labs on the classification of the TTC-stained seeds, particularly concerning the TTC-state "intermediate", in a second step the experimental set-up of the joint trial was extended by further germination/TTC test and stand-alone TTC test to: 4) obtain a precise adjustment of the testing method, as well as a tight guideline for interpreting the results, particularly for the TTC state "intermediate" because a proper classification of these seeds is still a challenge when determining viability rates.

Material and methods

Joint trial (test comparison by the SMARTER team)

Plant material

Mature, dry seeds of common ragweed were collected from the years 2011 to 2014 on four different sites in Hungary and Austria (Table 1).

Immediately after collection, seeds were dried at room temperature, air purified and placed at 4 °C ± 2 °C in a dark refrigeration chamber until the beginning of the experiment. Eight institutions participated in the joint trial which started in 2015 (Table 2).

Table 1. Locations, year of collection, coordinates, habitat type and 100 kernel weight of the two Hungarian and two Austrian seed origins of common ragweed analysed in the joint trial.

Population	Year of collection	Coordinates	Habitat type	100 kernel weight (mean ± std)
Hungary 1 (H1-2011) Kaposvar	2011	46°22'07.70"N, 17°51'07.90"E	arable field	3.621 ± 1.128
Austria 1 (A1-2012) Hagenbrunn	2012	48°19'56.90"N, 16°24'21.77"E	ruderal area	5.424 ± 1.642
Austria 2 (A2-2013) Seyring	2013	48°19'55.96"N, 16°29'15.04"E	ruderal meadow	4.778 ± 2.065
Hungary 2 (H2-2014) Kaposvar	2014	46°22'06.30"N, 17°50'59.50"E	arable field	3.565 ± 1.292

Table 2. Participating institutions in the joint trial.

Institution	Country	Number of testers
University of Natural Resources and Life Science Vienna (AT)	Austria	2
Czech Academy of Science, Pruhonice (CZ)	Czech Republic	1
Julius Kuehn-Institut Braunschweig (D)	Germany	1
Kaposvár University (H)	Hungary	1
NL Food and Consumer Product Safety Authority (NL)	Netherlands	2
Babeş-Bolyai University, Cluj (RO)	Romania	2
University of Novi Sad (SRB)	Serbia	1
Düzce University (TR)	Turkey	1

2,3,5-triphenyltetrazolium chloride (TTC) test procedure

The TTC assay is a fast evaluation for seed viability. Respiring tissues are capable of converting a colourless compound to a carmine-red coloured water-insoluble formazan by hydrogen transfer reaction, catalysed by the cellular dehydrogenase. TTC enters both living and dead cells, but only living cells catalyse the formazan, resulting in colouration of these tissues (Moussa et al. 2013).

For the first run of TTC testing, 50 intact seeds from each locality (Austria 1 + 2 and Hungary 1 + 2) were selected and soaked with tap water for 12–15 hours. After soaking, the seeds were cut with a medical scalpel longitudinally into two halves and the presence of the embryo was checked using a microscope. Seeds with obviously intact embryos were placed in 0.5 ml PCR-tubes that were filled with 1%-TTC in demineralised water and were incubated for 24 hours at 30 °C in darkness. Afterwards, the embryos of the seeds were checked under a microscope to determine if there was a discolouration according to the TTC-staining-protocol provided by COST ACTION FA1203 (following Starfinger and Karrer 2016), which determines the three different categories "alive", "intermediate" and "dead" (Fig. 1, Table 3). According to this protocol, completely discoloured seeds are classified as fully viable, but behaving as dormant, completely non-coloured seeds are classified as dead, since there are no living cells, which would have changed colour when treated with TTC and partial discoloured seeds are seen to be in an intermediate stage. The same procedure with again 50 seeds from each locality was repeated in a second run, which was executed by another independent observer. In case of the labs AT, NL and RO, the staining of all tested seeds was evaluated twice, namely by two different observers independently.

In the joint trial, it became obvious that especially the number of non-viable classified seeds per seed lot varied heavily between labs due to the circumstance that these

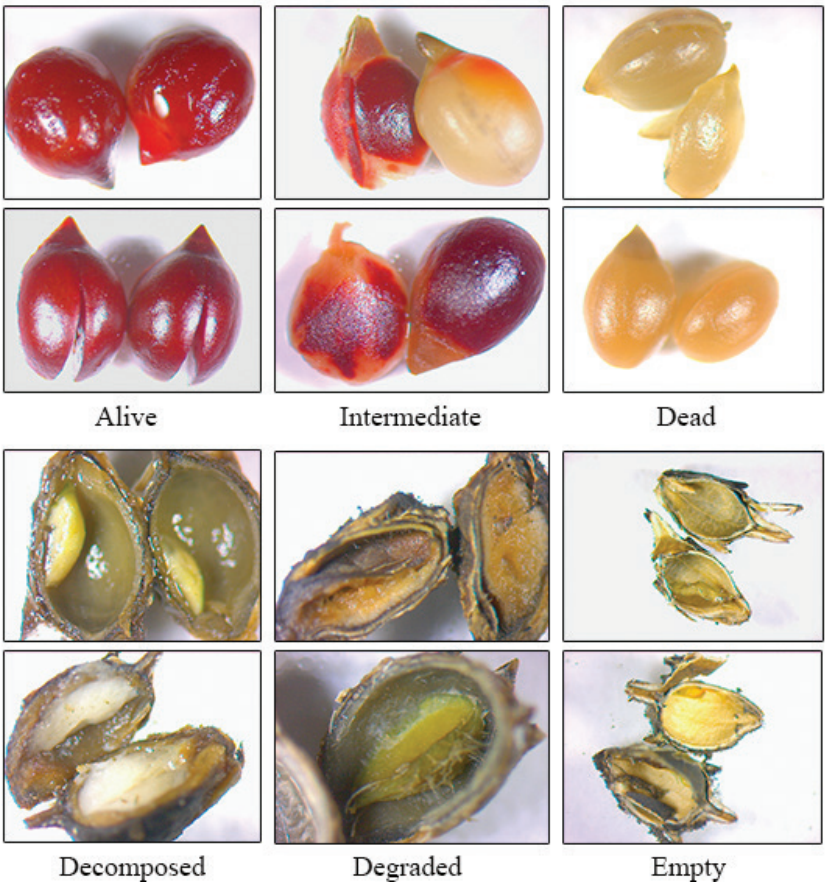

Figure 1. The six classification categories of seeds in the TTC test.

Table 3. Description of the possible TTC states in TTC test.

Viability status	Description
positive "alive"	both halves of the embryo are completely, deep carmine-red-coloured
intermediate	partial discolouration on the halves of the embryo
negative "dead"	both halves of the embryo show no discolouration
decomposed	all seeds in which the embryo showed severe decomposition
degraded	seed which had dried out and had already started to decay
empty	seeds in which the embryo did not develop or had completely decayed

seeds were not only obviously dead (no discolouration in TTC) or empty (Karrer et al. 2016b). Thus, for the extended trial, two more state categories (decomposed, degraded) were introduced to avoid bias in the results (Fig. 1, Table 3). Those decomposed or degraded seeds had an embryo and also showed discolouration, but these embryos had deformations, showed indications for drying-out-effects or liquefaction. Thus, the seeds with decomposed or degraded embryos were calculated in the sum of TTC-negative tested seeds.

Germination test

In a first run, the test was performed on 50 seeds from each of the seed lots Austria 1 and Austria 2, as well as of Hungary 1 and Hungary 2. Stratification of the first set of seed origins was done in Petri dishes with 9 cm diameter, which were filled with fine quartz sand (0.1–0.7 mm grain size), building a layer of approximately 0.5 cm thickness. After placing the unsterilised seeds (17, 17, 16 seeds per dish) on the surface of this quartz sand layer, 10 ml tap water was added, Petri dishes were closed with the upper shell and seeds were stratified at 4 °C for six weeks in darkness. This procedure was repeated approximately two weeks after the first run with a second set of 50 seeds from each locality, respectively.

After six weeks of stratification, the Petri dishes were moved to a climate chamber with 12 hours full light at 25 °C and 12 hours darkness at 15 °C (optimum conditions for ragweed germination defined by Leiblein-Wild et al. 2014 and Farooq et al. 2019) and incubated for 28 days. Petri dishes were checked three times per week. Seeds with a visible radicle were recorded as germinated and removed (Karrer et al. 2016c). Seeds which did not germinate within the 28-days-period were afterwards tested with TTC following the same protocol as mentioned above, except for soaking, due to the fact that seeds were continuously kept wet during the germination test. Fully coloured TTC-tested seeds were counted as viable together with the germinated seeds.

Crush test

From each location and year, 100 intact seeds were cut longitudinally into two halves. The larger half seed was placed on a filter paper on a glass slide with the cut side touching the paper. Each seed half was crushed by placing another glass slide on top and pressing firmly all the way down to the paper. When the seeds caused liquid staining after crushing on the filter paper, the seed was considered alive. If the filter paper was dry or the external intact seed was empty, the seed was considered dead (Karrer et al. 2016c).

Extended trial

The extension of viability tests was executed in the BOKU lab only. In the extended trial, mature dry seeds of common ragweed were randomly selected from twelve populations, which were harvested from the years 2010 to 2014 from ten plants growing on twelve different sites in south-eastern parts of Austria (Table 4). Immediately after collection, seeds were dried at room temperature, air purified and placed at 4 °C in a refrigeration chamber until the beginning of the experiment. Germination tests plus subsequent TTC tests, as well as a stand-alone TTC test, were performed on parallel subsamples of the seed lots following the same protocol as with the joint trial. For each test, 300 obviously intact seeds of each of the twelve populations were randomly selected. A total of 100 of them were weighed and measured for length and width.

Table 4. Locations, year of collection, coordinates and habitat type of the 12 Austrian seed origins of common ragweed analysed in the extended-trial.

Location	Pop. code	Year of collection	Coordinates	Habitat type
Seyring	SEY	2014	48°19'55.96"N, 16°29'15.04"E	ruderal site
Hartberg	HAR	2013	47°16'53.44"N, 15°58'22.91"E	roadside
Fürstenfeld	FÜF	2013	47°2'53.55"N, 16°4'48.76"E	roadside
Halbenrain	HAL	2013	46°43'20.95"N, 15°56'50.93"E	arable field
Neunkirchen	NEK	2013	47°43'33.96"N, 16°4'52.26"E	arable field
Sankt Pölten	STP	2013	48°12'12.96"N, 15°38'18.44"E	roadside
Zillingtal	ZIL	2012	47°47'12.93"N, 16°26'47.61"E	arable field
Leobendorf	LEO	2012	48°22'31.92"N, 16°19'32.75"E	arable field
Neue Donau	NDO	2012	48°12'59.68"N, 16°25'45.84"E	ruderal site
Deutsch Wagram	DWA	2010	48°17'56.59"N, 16°33'50.44"E	roadside
Unterpurkla	UPU	2010	46°43'54.48"N, 15°54'11.30"E	arable field
Hagenbrunn	HAG	2010	48°19'56.90"N, 16°24'21.77"E	ruderal site

Furthermore, various studies showed that the carbon/nitrogen-ratio (C/N-ratio) has a severe impact on the seed viability and their ability to germinate. For example, medium levels of maternal nitrogen (N) led to medium N-levels in the offspring, which subsequently accelerated germination. Additionally, it was observed that nitrate provided by the mother plant acts as a signal molecule to seed dormancy breakage (Holdsworth et al. 2008; Karimmojeni et al. 2014). Thus, the average nitrogen (N) and carbon (C) concentration and subsequently the C/N-ratio of 50 randomly selected seeds per population was determined by the Dumas Combustion Method (Winkler et al. 2000), using an elemental analyser (vario MAX cube CNS, Elementar Analysesysteme GmbH, Germany). This procedure was replicated for all seed origins 10 times (n = 5,000 seeds per population).

Data analysis

Germination rate

The final germination rate (germinated versus non-germinated seeds) was used as a primary dependent variable for analysis.

Mean germination time

The mean germination time (MGT) is a dimensionless indicator of the germination performance, opposing the germination rate and the temporal distribution of germination of each single seed and is calculated according to Ellis and Roberts (1980) as follows:

$$MGT = \Sigma (t \times n) / \Sigma n,$$

t – time in days; n – is the number of seeds which completed germination on day t. Lower MGT values indicate faster germination.

Statistical analysis

Sigma Plot 12.5 was used for graphical visualisation of the results. Statistical analyses were performed using software SAS version 9.2. Analysis of variance (PROC GLM) was used to test the influence of the independent factors origin, age and testing lab on germination rate and/or seed viability. Subsequently, multiple comparisons of means according to Student-Newman Keuls were performed. Means were separated by least significant differences (LSD), when the F-test indicated factorial effects on the significance level of $p < 0.05$. The Shapiro Wilk test was used to test the normal distribution of data and Levene's test was used to check equality of variances. If normal distribution were not given, a Kruskal-Wallis ANOVA on Ranks was performed. If homogeneity of variances were not given, statistical analysis was executed, using Welsh's test of unequal variances t-test.

Logistic regression analyses (PROC LOGISTIC) was performed to evaluate the significance of the explanatory factor origin and sampling year. Due to sufficient replications, particularly in the extended joint trail, we further tested if there is significant influence of the habitat type on the results. Linear regressions models (PROC REG) were used to test the influence of all the factors on seed viability and on the different viability states gained with TTC testing.

In the extended trial, randomized samples for the germination test and subsequent TTC test, as well as for the stand-alone TTC test, were drawn from twelve different populations. One prerequisite when testing the viability of seeds with different testing methods is homogeneity of samples. To check if the randomised samples for germination test and subsequent TTC test, as well as for the stand-alone TTC test, are comparable, a Chi^2-test was performed. Since only embryo-bearing seeds can be viable (able to germinate or TTC-stained), the parameter "intact embryo" was used as the indicator to evaluate the probability of samples deriving from same population.

Probability of an intermediate stained seed to be viable

TTC-positive and TTC-negative seeds are quite easy to determine, but the intermediate state covers a wide range of different colouration intensities. Since germination tests with TTC-treated seeds are not possible, a statistical analysis (Chi^2-test) on the basis of the results of germination test and TTC test was performed to calculate the probability of a TTC-intermediate tested seed being viable or not.

Results

Joint trial

Germination rate

Germination was tested by all eight labs participating in the joint trail. The Turkish lab (TR) only reported results on the germination rate. Information on the mean germination time was not submitted.

The origin and the sampling year had a significant impact (age; F = 19.89; p < 0.001) on the germination rate of common ragweed seeds (Fig. 2). Interaction effects of these two factors were not significant (F = 0.316; p = 0.579). In all laboratories participating within the joint trial, H1-2014 showed the highest germination rate which accounted for 74.7% on average, followed by the population A2-2013 with a mean of 69.4%. These two younger seed lots differed significantly from the older seed lots A1-2012 and H2-2011, the latter accounting for an average germination rate of 36.5% and 25.4%, respectively (Table 5). Even though the results varied widely between labs, the factor lab had no significant influence (ANOVA: F = 0.948; p = 0.483) on the germination test results (Table 5).

Mean Germination Time (MGT)

The year of harvest (age) had a significant impact on the MGT of the seeds of common ragweed (F = 174.76; p < 0.001, Table 5, Suppl. material 1: Fig. S1). The fastest germination activity of 0.118 on average was observed with the seeds of A2-2013, deriving from a ruderal meadow north of Vienna. A total of 50% of the seeds of this population germinated within the first eight days after incubation. In contrast, the seeds from A1-2012 took the longest incubation time before germination with an MGT of 0.449 (median: 11 days after trial start). Contrary to the germination rate, the MGT varied widely amongst labs, even though all participants followed the same

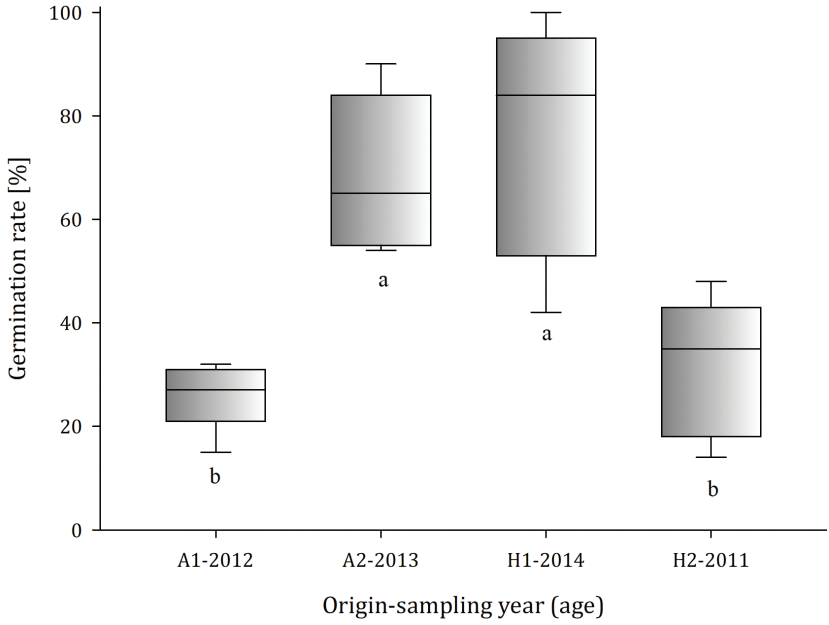

Figure 2. Germination rate [%] of common ragweed seeds in dependency of the factors origin and year (n = 700/population/year ; Two-way-ANOVA, different letters indicate significant differences).

Table 5. Results of the eight independent laboratories for the germination rate [%], mean germination time, share [%] of ragweed seeds tested alive, intermediate and dead in stand-alone TTC test, as well as the share [%] of common ragweed seeds tested alive with crush-test, with regard to the four different origins of the seeds (A1-2012, A2-2013, H1-2014, H2-2011; two numbers in the columns show the result of two individual testers).

	Austria (AT)	Czech Rep. (CZ)	Germany (D)	Hungary (H)	Nether-lands (NL)	Romania (RO)	Serbia (SRB)	Turkey* (TR)	overall mean
A1-2012									
Germination test									
Germination rate [%]	31	21	15	27	29	23	32	40	**25.4**
MGT	0.251	0.370	0.822	0.209	0.314	0.518	0.660	–	**0.449**
Stand-alone TTC test									
positive [%]	19 | 21	3	29	6	27 | 24	29 | 36	12	8	**19.5**
intermediate [%]	25 | 20	21	8	20	6 | 9	50 | 45	0	12	**19.6**
dead [%]	56 | 59	76	63	74	67 | 67	21 | 19	88	80	**60.9**
Crush-test: alive [%]	94	97	100	70	98	98	78	100	**91.9**
A2-2013									
Germination test									
Germination rate [%]	64	74	65	55	54	90	84	74	**69.4**
MGT	0.072	0.065	0.154	0.087	0.133	0.121	0.197	–	**0.118**
Stand-alone TTC test									
positive [%]	54 | 47	46	75.0	26.0	76 | 78	61 | 67	60	23	**55.7**
intermediate [%]	26 | 30	37	8.0	40.0	6 | 4	28 | 21	0	20	**20.0**
dead [%]	20 | 23	17	17.0	34.0	18 | 18	11 | 12	40	57	**24.3**
Crush-test: alive [%]	89	99	95	54	94	99	100	97	**90.9**
H1-2014									
Germination test									
Germination rate [%]	84	91	53	42	58	95	100	76	**74.7**
MGT	0.087	0.115	0.301	0.132	0.192	0.157	0.172	–	**0.165**
Stand-alone TTC test									
positive [%]	76 | 84	74	86	36	90 | 94	85 | 92	86	64	**78.8**
intermediate [%]	22 | 14	15	0	28	7 | 2	12 | 5	2	21	**11.6**
dead [%]	2 | 2	11	14	36	3 | 4	3 | 3	12	15	**9.6**
Crush-test: alive [%]	97	82	91	66	95	96	96	100	**90.4**
H2-2011									
Germination test									
Germination rate [%]	28	35	14	43	18	48	70	36	**36.5**
MGT	0.281	0.260	0.969	0.099	0.623	0.336	0.243	–	**0.402**
Stand-alone TTC test									
positive [%]	36 | 37	29	62	20	43 | 66	56 | 62	54	29	**44.9**
intermediate [%]	33 | 30	51	26	28	28 | 9	37 | 33	0	13	**26.2**
dead [%]	31 | 33	20	12	52	29 | 25	7 | 5	46	58	**28.9**
Crush-test: alive [%]	84	88	97	56	93	98	98	96	**88.8**

* the Turkish lab only reported the results for the germination rate; information on the mean germination time was not available.

incubation protocol. However, statistical analysis showed that the various labs had significantly different results ($F = 22.4$; $p < 0.001$; Fig. 3). Except for A2-2013, all other seeds which were tested in the German lab showed a significantly higher MGT than all other laboratories. On average, in this lab, it took 14 days until 50% of the ragweed seeds germinated, whereas in the Hungarian lab, it took only 4 days until half of the seeds germinated. Similar results were observed with seeds tested in the Romanian, the Dutch and the Serbian laboratory, respectively, which all showed significantly higher MGT than all other labs, particularly with seed lots A2-2013, H1-2014 and H2-2011.

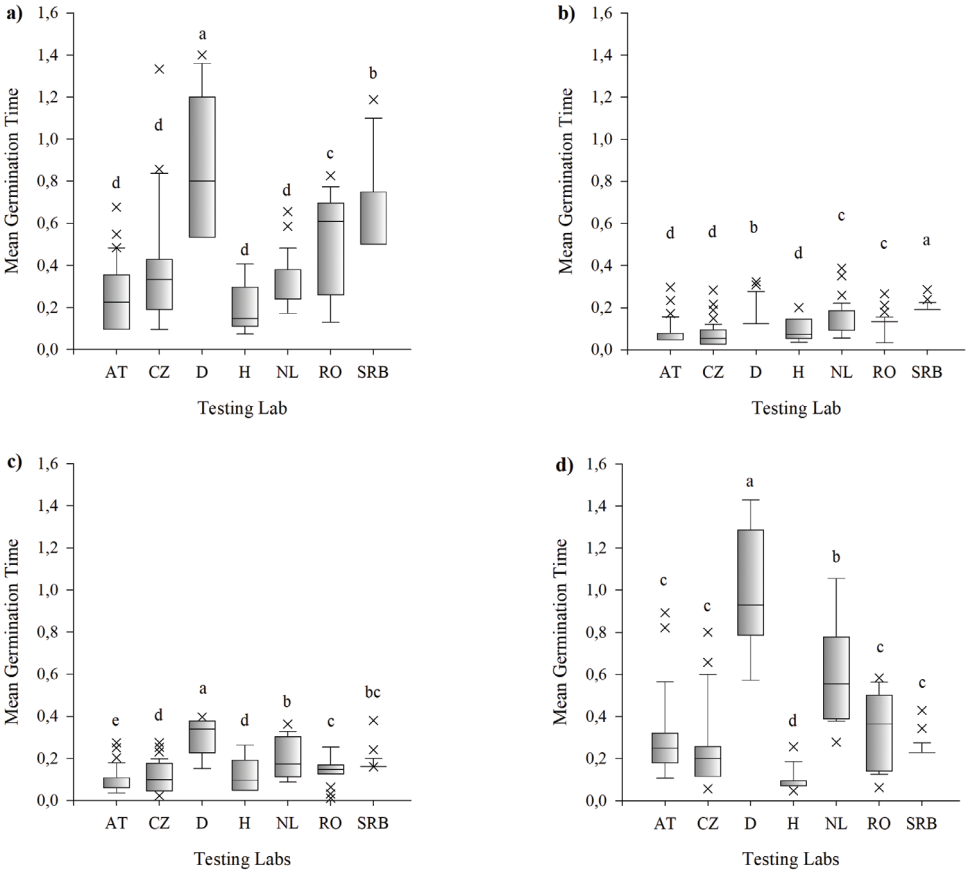

Figure 3. Mean germination time of ragweed seeds from populations **a** A1-2012 **b** A2-2013 **c** H1-2014 and **d** H2-2011 in relation to the factor testing lab (n = 700 seeds/population; 100 seeds/lab; different letters indicate significant differences); for country codes, see Table 5.

Stand-alone TTC test

As with germination, the factors age and origin ($F = 28.36$; $p < 0.001$), respectively, had significant impact on the results of the stand-alone TTC test within the joint trial (Table 5, Suppl. material 1: Fig. S2). On average, H1-2014 had a share of 78.8% TTC-positive tested seeds. A total of 9.6% of these seeds were classified as TTC-negative and 11.6% were classified as intermediate. The seeds A2-2013 showed on average, over all laboratories, a share of 55.7% viable seeds and 20.0% were classified as intermediate. Even though H2-2011 were the oldest from all samples, the share of TTC-positive tested seeds accounted for 44.9% and was, therefore, significantly higher than with the seeds of A1-2012 which had the significantly lowest share of TTC-positive tested seeds (19.5%), as well as the significantly highest share of TTC-negative tested seeds (60.9%).

Variation amongst labs

Significant differences in TTC-test results were also observed amongst labs (Fig. 4). Especially with the seed lots A2-2013, H1-2014 and H2-2011, the Hungarian lab had the significantly lowest share of viable seeds, if viable seeds consist of TTC-positives only, as well as in the case of intermediates being included into the group of viable seeds. The highest average number of viable (= TTC-positive tested) seeds (65.5%) was observed by tester 2 of the Dutch lab, but this did not differ significantly neither from the results of the first Dutch tester nor from the results of various other labs. Particularly with the TTC-state "intermediate", the Serbian lab observed the significantly lowest share of intermediate seeds with 0.5%. Especially with populations A1-2012, A2-2013 and H2-2011, not a single seed was classified as intermediate with the Serbian lab. The highest share of intermediate stained seeds of 31.8% was measured by Romanian tester

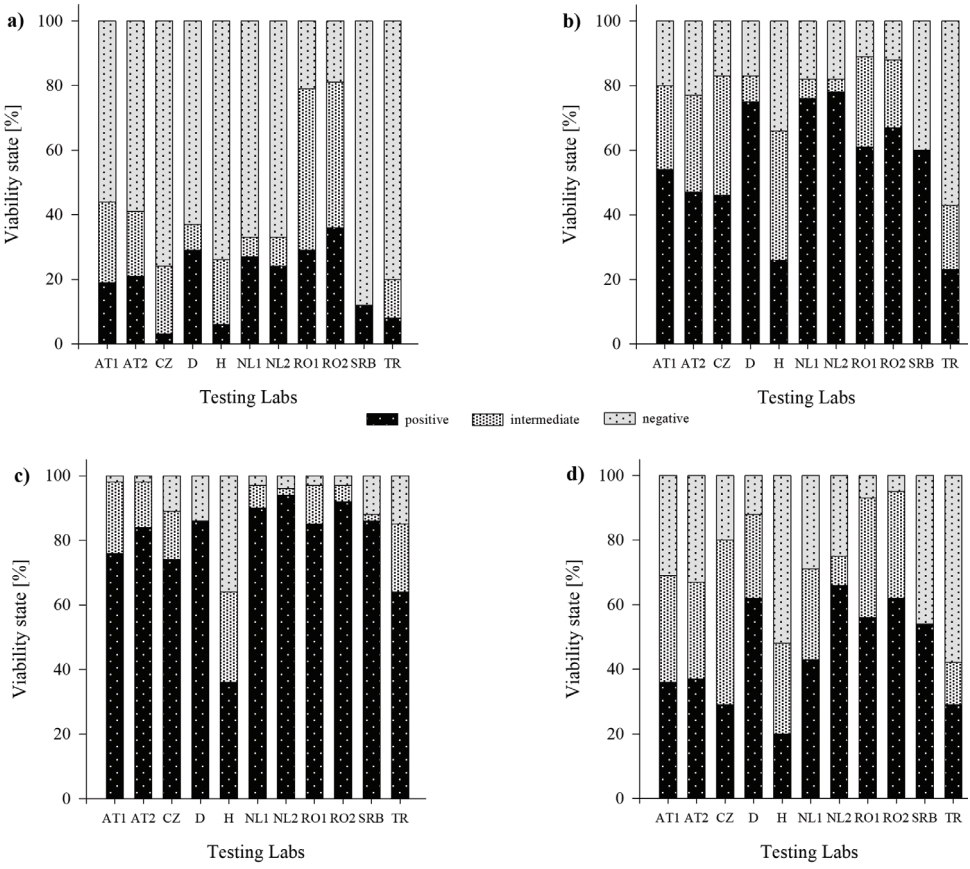

Figure 4. Relative frequency [%] of the viability states of ragweed seeds in the stand-alone TTC test depending on the testing laboratory for **a** population A1-2012 **b** A2-2013 **c** H1-2014 and **d** population H2-2011. In Austria (AT1 and AT2), The Netherlands (NL1 and NL2) and Romania (RO1 and RO2), seeds were tested by two individual testers (n = 4,400).

1, but this result was not significantly higher than some other labs. In contrast, testers Romania 1 and Romania 2 had the significantly lowest share of TTC-negative classified seeds within their samples (Suppl. material 1: Table S1).

Crush-test

As shown in Table 5 and Fig. 5, the crush test led to a significant overestimation of viability of ragweed seeds in five out of eight labs, irrespective of the year of sampling (age) and/or origin of seeds. Only in the Hungarian lab, the number of viable seeds tested with the crush test was comparable with the results of the germination test. On a lab level, the highest discrepancy amongst the germination test, TTC test and crush test was observed in the Turkish lab, where the crush test showed an overestimation of 55.6% on average compared to both the other testing methods. With respect to the populations, the highest average discrepancy in results was observed with ragweed population A1-2012: On average, 25.4% of the seeds germinated and 19.5% were tested positive in the stand-alone TTC test, but with the crush test, a viability rate of 91.9% was computed.

The Extended Trial

Table 6 summarises the results of germination test and subsequent TTC test, as well as stand-alone TTC test confirming the results of the joint trial as we found significant impact of the factors origin and age (year of sampling) on the germination rate (Fig. 6 and Suppl. material 1: Fig. S3), viability rate and mean germination time of ragweed seeds.

The highest mean germination rate of 96.0% was observed with seeds from Seyring (SEY) which had also the shortest MGT of 0.05, whereas the lowest germination rate of 3.0% on average was shown by the seeds deriving from Hagenbrunn (HAG) which showed the highest MGT of 1.33 (Table 6; Fig. 6b; Suppl. material 1: Fig. S4).

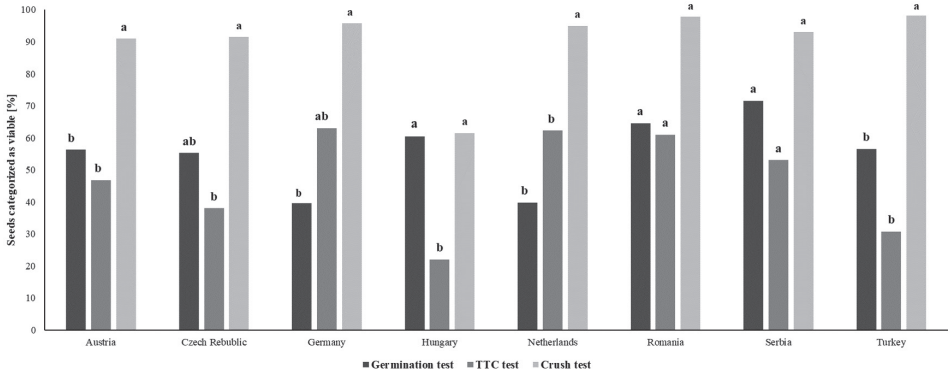

Figure 5. Percentage of seeds tested as viable with the germination test, stand-alone TTC test and crushtest on average over four populations depending on the factor testing lab (n = 300 per population and testing lab; different letters indicate significant differences).

Table 6. Germination rate [%] and the share of seeds [%] tested as viable, intermediate or non-viable in either germination test plus subsequent TTC test or stand-alone TTC test, the mean germination time (2-tailed t-test, n = 7,200; superscript letters indicate significant differences between the respective values from different seed origin or from different sampling years, respectively).

Origin	Germination test + subsequent TTC test				Stand-alone TTC test			Mean germination time
	Germination rate [%]	TTC-positive [%]	TTC-intermediate [%]	TTC-negative* [%]	TTC-positive [%]	TTC-intermediate [%]	TTC-negative* [%]	
Seyring (SEY)	96.0 ^{a}	1.5	0.0	2.5	87.0	1.5	11.5	0.046 ^{a}
Hartberg (HAR)	74.0 ^{b}	4.0	3.5	18.5	66.0	15.0	19.0	0.072 ^{ab}
Fürstenfeld (FUF)	63.5 ^{c}	9.5	5.0	22.0	40.0	37.0	23.0	0.096 ^{ab}
Halbenrain (HAL)	75.0 ^{b}	7.0	6.5	11.5	68.0	13.5	18.5	0.066 ^{ab}
Neunkirchen (NEK)	53.5 ^{c}	14.0	12.0	20.5	48.5	23.5	28.0	0.151 ^{abc}
Sankt Pölten (STP)	36.0 ^{de}	12.0	25.0	27.0	26.0	42.5	31.5	0.216 ^{c}
Zillingtal (ZIL)	22.5 ^{f}	16.0	29.0	32.5	6.0	57.0	37.0	0.418 ^{d}
Leobendorf (LEO)	44.0 ^{d}	10.0	21.5	24.5	19.0	38.0	43.0	0.175 ^{bc}
Neue Donau (NDO)	25.0 ^{ef}	10.0	41.0	24.0	22.5	36.5	41.0	0.253 ^{c}
Dt. Wagram (DWA)	16.5 ^{f}	8.5	20.5	54.5	11.5	56.5	32.0	0.487 ^{d}
Unterpurkla (UPU)	7.0 ^{g}	1.0	30.0	62.0	2.5	29.5	68.0	0.796 ^{e}
Hagenbrunn (HAG)	3.0 ^{g}	1.5	33.5	62.0	0.0	34.0	66.0	1.333 ^{f}
Year								
2014	96.0 ^{a}	1.5	0.0	2.5	87	1.5	11.5	0.046 ^{a}
2013	60.4 ^{b}	9.3	10.4	19.9	49.7	26.3	24.0	0.120 ^{b}
2012	30.5 ^{c}	12.0	30.5	27.0	15.8	43.8	40.3	0.282 ^{c}
2010	8.8 ^{d}	3.7	28.0	59.5	4.7	40.0	50.3	0.872 ^{d}

*Seeds with degraded or decomposed embryo were calculated in the sum of TTC-negative tested seeds.

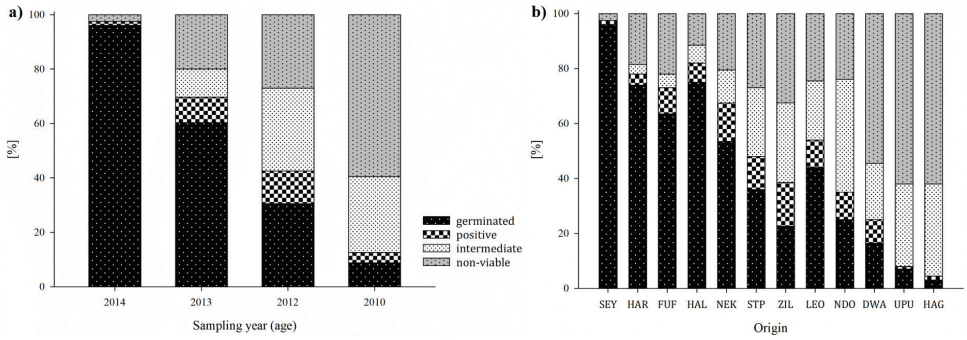

Figure 6. Germination rate [%; black-dotted bars) of ragweed seeds from 12 Austrian origins, as well as the share of positive, intermediate and negative tested ragwed seeds in subsequent TTC tests in relation to the factors **a** age (year of harvest) and **b** origin (n = 3,600); seeds with degraded or decomposed embryos were calculated in the sum of TTC-negative tested seeds.

A regression analysis revealed that the ability to germinate and viability of the tested ragweed seeds was tightly correlated with the origin ($R^2 = 0.91$; $F = 99.17$; $p < 0.001$) and the year of harvest ($R^2 = 0.97$; $F = 65.57$; $p < 0.05$), respectively. Within years, only the seeds from Sankt Pölten (STP; 2013) showed a significant lower number of germinated seeds, but when adding the TTC-positive tested embryos to the germinated seeds (= viable) also within the year 2013, no significant differences between the origins were observed.

With the stand-alone TTC test, similar results were obtained as with the germination test (Table 6; Fig. 7). The number of positive-tested seeds was correlated with the origin ($R^2 = 0.82$, $F = 45.49$; $p < 0.001$) and the age ($R^2 = 0.84$; $F = 26.27$; $p < 0.05$), respectively. This is also true for the TTC-negative tested seeds; their number increased significantly with age ($R^2 = 0.99$, $F = 754.33$; $p < 0.001$) and was also related to the origin of the populations ($R^2 = 0.81$, $F = 43.52$; $p < 0.001$). In contrast to germination testing, it should be noted that the TTC-positive tested seeds showed generally much lower viability rates, whereas the share of intermediate coloured seeds was significantly higher (Fig. 8).

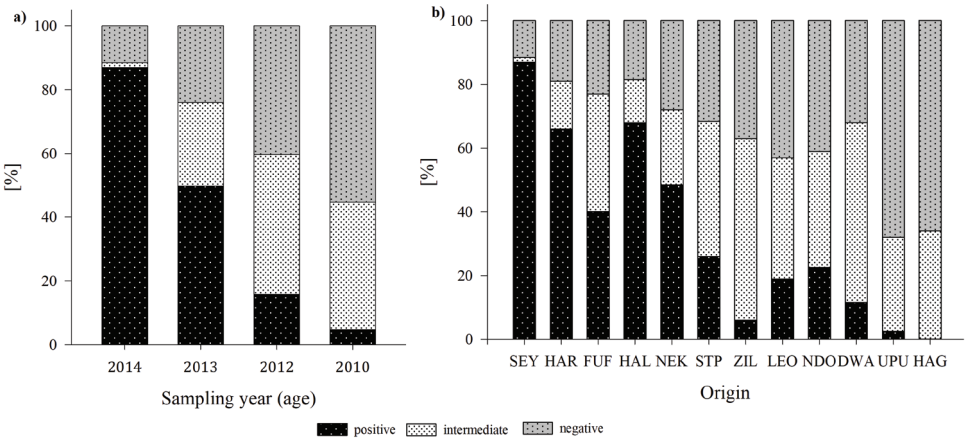

Figure 7. Share of ragweed seeds (%) tested positive, intermediate and negative in the stand-alone TTC test in relation to the factors **a** age (year of harvest) and **b** origin (n = 3,600).

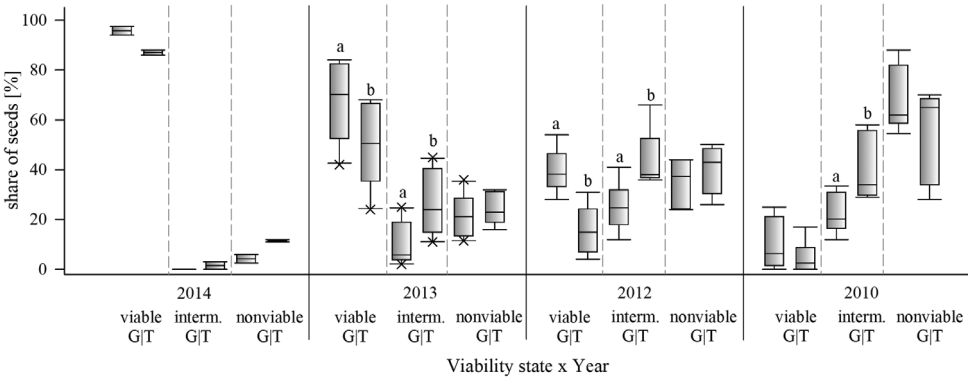

Figure 8. Results gained for the viability status of ragweed seeds estimated "viable" either by the germination test plus positive subsequent TTC test (G) or by positive stand-alone TTC test (T) dependent on the year of sampling (age); different letters indicate signficant differences between the testing methods, missing letters indicate no significant differences (2-tailed t-test; n = 7,200).

Seed weight, seed size and carbon/nitrogen-ratio (C/N-ratio)

As summarised in Table 7, we found significant differences in the seed weight of common ragweed with dependency on the factors of origin and age. The seed size (length × width) which was almost similar amongst populations did not have any impact on the results. Seeds deriving from SEY (harvested in 2014) showed an average weight of 8.8 mg ± 2.1. This was significantly higher than those of all other populations, expect that of ZIL (harvested in 2012). With an average weight of 4.7 mg ± 1.7, the lightest seeds derived from UPU (harvested in 2012). Similar seed weights were measured before the stand-alone TTC test. Results of logistic regression revealed that the factors of weight and year had significant influence on the germination rate and the viability states (stand-alone TTC test) of common ragweed seeds. However, with the germination test, we could not find any correlation between seed weight and ability to germinate. Although the youngest and heaviest seeds from SEY (2014) also showed the significantly highest germination rate, we could not detect any similar pattern amongst the other populations. For example, the average weight of common ragweed seed from ZIL (2012) was 8.0 mg ± 1.5 which did not differ from that of SEY, but the germination rate of these seeds was 73.5% lower. Similar results were obtained for the stand-alone TTC test. The highest share of positive-tested seeds (87%) was observed with seeds from SEY which had an average weight of 9.0 mg ± 1.3. However, this did not differ significantly from

Table 7. Average weight (mean ± sd) and size of ragweed seeds used in the germination test and subsequent TTC test, as well as in stand-alone tests (n = 100 seed/population), as well as the average seed weight and seed size of germinated and TTC-positive, TTC-intermediate and TTC-negative tested ragweed seeds; superscript letters indicate significant differences.

Origin	Average seed weight [mg]	Average seed size (length × width [mm]	Average weight of germinated seeds	Average seed weight [mg]	Average seed size (length × width [mm]	Average weight of TTC-positive seeds	Average weight of TTC-intermediate seeds	Average weight of TTC-negative seeds
Seyring (SEY)	8.8 ± 2.1 ^{a}	3.5 × 2.3	9.1 ± 1.8 ^{a}	8.7 ± 2.5 ^{a}	3.9 × 2.2	9.0 ± 1.3 ^{a}	–	3.2 ± 1.8 ^{c}
Hartberg (HAR)	6.9 ± 1.6 ^{b}	3.1 × 2.5	7.1 ± 1.4 ^{b}	7.1 ± 1.8 ^{ab}	3.1 × 2.4	7.6 ± 1.1 ^{b}	8.0 ± 0.8 ^{a}	4.9 ± 2.3 ^{abc}
Fürstenfeld (FUF)	5.7 ± 1.2 ^{c}	3.0 × 2.0	5.5 ± 1.3 ^{c}	5.6 ± 1.5 ^{cd}	3.1 × 2.1	5.7 ± 1.0 ^{b}	6.0 ± 1.5 ^{b}	4.3 ± 1.7 ^{abc}
Halbenrain (HAL)	6.9 ± 1.5 ^{b}	3.3 × 2.2	6.7 ± 1.2 ^{b}	6.7 ± 2.0 ^{bc}	3.5 × 2.4	7.4 ± 1.5 ^{b}	6.7 ± 1.5 ^{ab}	3.9 ± 2.1 ^{bc}
Neunkirchen (NEK)	6.7 ± 2.2 ^{b}	3.1 × 2.2	7.2 ± 1.6 ^{b}	6.6 ± 2.3 ^{bc}	3.1 × 2.2	7.0 ± 1.9 ^{b}	6.9 ± 1.6 ^{ab}	4.2 ± 2.1 ^{abc}
Sankt Pölten (STP)	6.6 ± 1.7 ^{b}	3.2 × 2.3	7.1 ± 1.6 ^{b}	6.7 ± 1.8 ^{bc}	3.1 × 2.2	6.7 ± 1.7 ^{b}	7.4 ± 1.9 ^{ab}	6.3 ± 1.7 ^{a}
Zillingtal (ZIL)	8.0 ± 1.5 ^{ab}	3.4 × 2.2	8.1 ± 1.3 ^{b}	7.6 ± 1.9 ^{bc}	3.0 × 2.1	8.2 ± 1.4 ^{ab}	7.3 ± 1.5 ^{ab}	5.9 ± 2.0 ^{ab}
Leobendorf (LEO)	6.8 ± 2.2 ^{b}	3.3 × 2.2	7.5 ± 1.8 ^{b}	6.4 ± 2.4 ^{bcd}	3.0 × 2.1	6.0 ± 2.1 ^{b}	6.8 ± 1.4 ^{ab}	3.9 ± 2.5 ^{bc}
Neue Donau (NDO)	6.2 ± 1.6 ^{b}	3.1 × 2.1	6.9 ± 1.4 ^{b}	6.7 ± 1.9 ^{bcd}	3.0 × 2.1	5.4 ± 1.1 ^{b}	6.9 ± 1.2 ^{ab}	5.1 ± 2.2 ^{abc}
Dt. Wagram (DWA)	6.9 ± 1.6 ^{b}	3.2 × 2.2	6.9 ± 1.3 ^{b}	5.6 ± 2.5 ^{cd}	3.0 × 2.1	8.0 ± 0.6 ^{ab}	6.6 ± 2.1 ^{ab}	3.6 ± 3.8 ^{bc}
Unterpurkla (UPU)	4.7 ± 1.7 ^{d}	3.0 × 2.1	–	4.5 ± 1.7 ^{d}	3.2 × 2.1	–	6.1 ± 1.2 ^{b}	3.8 ± 1.5 ^{bc}
Hagenbrunn (HAG)	6.0 ± 1.8 ^{bc}	3.2 × 2.0	–	5.4 ± 1.9 ^{cd}	3.3 × 2.1	–	6.5 ± 1.3 ^{ab}	4.9 ± 1.9 ^{abc}
Year								
2014	8.8 ± 2.1 ^{a}	3.5 × 2.2	9.1 ± 1.8 ^{a}			9.4 ± 1.3 ^{a}	–	3.2 ± 1.8 ^{b}
2013	6.6 ± 1.7 ^{b}	3.2 × 2.3	6.8 ± 1.5 ^{b}	6.5 ± 2.0	3.2 × 2.3	7.1 ± 1.5 ^{b}	6.8 ± 1.7 ^{a}	4.8 ± 2.1 ^{a}
2012	7.0 ± 1.9 ^{b}	3.2 × 2.1	7.4 ± 1.6 ^{b}	6.5 ± 2.2	3.0 × 2.1	6.2 ± 1.8 ^{c}	7.0 ± 1.4 ^{a}	5.0 ± 2.4 ^{a}
2010	6.3 ± 1.9 ^{b}	3.1 × 2.1	6.9 ± 1.3 ^{b}	5.4 ± 2.1	3.2 × 2.1	7.9 ± 0.6 ^{b}	6.4 ± 1.7 ^{a}	4.2 ± 1.9 ^{a}

the average weight of seeds deriving from ZIL (8.2 mg ± 1.4) or DWA (8.0 mg ± 0.6) which only had a share of positive-tested seeds of 6% and 11.5%, respectively. The overall mean of all positive-tested seeds amounted to 7.1 mg ± 1.2 which was similar to that of the intermediate-tested seeds (6.9 mg ± 0.6). With 4.5 mg ± 0.9, the average seeds' weight of the TTC-negative tested seeds was significantly lower.

In addition, we could not find any differences in the C/N-ratio of the seeds, ranging between 9.4 (SEY) and 11.5 (HAL). With an $R^2 = 0.09$, a regression analysis pointed out that viability of common ragweed seeds could not be correlated to the C/N-ratio (results not shown).

Discrepancies in the results with respect to the testing method

The initial Chi²-test to check for homogeneity of samples showed no significant differences; hence, it can be assumed that all samples randomly taken derive from equal populations and are, therefore, comparable. This is also true amongst testing methods comparing the seed samples used in the germination test and subsequent TTC test plus the stand-alone TTC test (Suppl. material 1: Table S2). Thus, discrepancies in the results due to unparalleled samples could be excluded. However, we found significant differences between testing results.

Figure 8 shows a comparison between the results for the viability state of the ragweed seeds between viable due to the germination test plus the subsequent TTC test (classified positive) and viable due to the positive classification in the stand-alone TTC test, compiled with respect to sampling years. Both testing methods gave comparable results for the non-viable embryos (mean over all populations of non-viable seeds' germination test plus TTC: 30.1%; TTC test alone: 34.9%). The evaluation of "viable" (germinated + subsequently TTC-positive classified seeds) and "intermediate" seeds showed significantly different results. Over all populations on average, the viability rate of the seeds accounted for 50.9% with germination test plus subsequent the TTC test, but with the stand-alone TTC test, only 33.1% of the seeds were assessed as viable (= positive) which results in a 17.8% lower viability rate.

However, a similar gap between results (13.1%) was detected when comparing the TTC-intermediate tested seeds within the germination test plus the subsequent TTC test and stand-alone TTC test: whereas the share of intermediate-stained seeds accounted for 18.9% with the germination test plus the subsequent TTC test, almost twice as many seeds (32.1%) were classified as "intermediate" with the stand-alone TTC test. For example, the greatest discrepancies were observed with seeds deriving from Fürstenfeld (FUF) which showed a 33.0% higher viability rate during germination compared to the results of the TTC test. The same was shown with the seeds from Zillingtal (ZIL) which accounted for a 32.5% higher viability rate with the germination test. Nearly exactly opposite percentage values were calculated for the intermediate seeds of these two populations, amounting to 32% for seeds from Fürstenfeld (FUF) and 28% for seeds from Zillingtal (ZIL; Table 6).

When comparing the results for the year of harvest (Fig. 8), a similar gap of "viable" and "intermediate" classified seeds between the germination test plus the subsequent TTC test and stand-alone TTC test was observed: seeds harvested in 2013 showed 20.0% more viable seeds (germinated plus fully TTC-stained seeds, respectively) with the germination test plus the subsequent TTC test than with the stand-alone TTC test. In contrast, the stand-alone TTC test showed 21.2% more intermediate seeds than the germination test plus the subsequent TTC test. In 2012, the viability rates were generally lower, but showed the same tendency: the rate of "viable" seeds was 26.7% higher with the germination test plus the subsequent TTC test, whereas the stand-alone TTC test accounted for 31.8% more intermediate seeds.

The seed staining state "intermediate"

The above figures indicate some discrepancies in the validity of "intermediate" state. It was not evident from the above figures if some of the seeds classified "intermediate" by a TTC test might be able to possibly germinate after the duration of the germination experiment. Table 8 summarises the results of the statistical analysis to calculate the probability on an intermediate stained seed to be viable (= able to germinate) or not. This analysis was performed on the basis of the results of the germination test plus the subsequent TTC test and stand-alone TTC test of the twelve Austrian seed lots of the extended trial. It should indicate that the probability of intermediate seeds to be viable is evident in eleven out of twelve populations independent of their age and

Table 8. Probability of ragweed seeds of intermediate status to be viable or non-viable verified by a Chi^2 -test: Col. 2: comparison number of viable (germinated + TTC-positive) seeds with the number of non-viable seeds without consideration of intermediate seeds; Col. 3: comparison of numbers of viable seeds and non-viable seeds, intermediate seeds were counted as non-viable; Col. 4: comparison of number of viable and non-viable seeds, intermediate seeds were counted as viable (n = 7,200); significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate probability if seed is viable or not.

Population	p-valuesfor the similarity of results of the germination test plus subs. TTC test and stand-alone TTC test (without intermediate seeds)	p-value for the probability of stand-alone TTC-tested intermediate seeds to be non-viable	p-values for the probability of TTC-intermediate seeds to be viable
Seyring (SEY)	0.05296	0,02753 *	0,05700
Hartberg (HAR)	0.26399	0.01625 *	0.48152
Fürstenfeld (FUF)	0.28149	< 0.001 ***	0.74054
Halbenrain (HAL)	0.14885	0.01366 *	0.26280
Neunkirchen (NEK)	0.29086	0.00901 **	0.90559
Sankt Pölten (STP)	0.13935	0.00499 **	0.87951
Zillingtal (ZIL)	< 0.001 ***	< 0.001 ***	0.85522
Leobendorf (LEO)	0.00119 **	< 0.001 ***	0.15576
Neue Donau (NDO)	0.03178 *	0.00935 **	0.30658
Dt. Wagram (DWA)	0.83300	0.03839 *	< 0.001 ***
Unterpurkla (UPU)	0.64976	0.54974	0.27286
Hagenbrunn (HAG)	0.08548	0.06912	0.44658
OVERALL	**0.26142**	**0.02538** *	**0.92871**

origin. Comparing the viability statistics over all populations, the probability of a seed classified as intermediate to be viable is 92.8% in this series of experiments (Col. 4). If intermediate seeds were not considered (Col. 2), particularly for the populations from Zillingtal (ZIL), Leobendorf (LEO) and Neue Donau (NDO), a great divergence between the results gained in the germination test plus the subsequent TTC test and in the stand-alone TTC test was observed, due to the highest number of intermediate seeds in just these populations. However, if these intermediate seeds were counted as viable, the results showed that the probability of these seeds to be viable is significantly higher than being non-viable. However, with increasing age, this classification was biased. All intermediate seeds that derived from populations harvested in the year 2010 (DWA, UPU, HAG) could not be classified since the probability of an intermediate seed to be viable or not was indifferent within this populations.

Discussion

Germination and Viability

The results of both trials showed that origin and age had a significant impact on the viability of ragweed seeds, irrespective if tested with the germination test or TTC test. In the joint trial, the youngest seeds H1-2014 showed a germination rate of 74.7%, which was twice as high as those of H2-2011. In addition, the share of TTC-positive tested seeds was 78.8%, almost double that of the seeds harvested in 2011 (44.9%).

Similar results were obtained with the extended trial - age and origin of the ragweed seeds influenced germination rate significantly. Results showed clearly that, with increasing age, the germination capacity declined sharply from 96.0% to 8.8% within 5 years, the share of viable TTC-tested seeds decreasing from 87.0% to 4.7% (2014 vs. 2010).

A distinct decrease in the viability of common ragweed seeds has already been proved by Karrer (2016) and Kazinczi and Kerepesi (2016), based on seeds stored at 4 °C or at room temperature, respectively. Our results are in accordance with Harrison et al. (2007) who investigated the demise of the seeds of a congener of common ragweed, *Ambrosia trifida* (giant ragweed) under cold dry storage. In this study, the germination capacity also declined from approximately 70% to 0–19% within a four years range.

Furthermore, significant differences within years and between the different origins, respectively, were observed with both testing methods. Thus, only seeds collected in the same year should be used for analysing effects of other factors.

Even though numerous studies already showed that traits like seed weight and seed size could play a vital role in germination behaviour of various plant species (Souza and Fagundes 2014; Kumar et al. 2017; Yi et al. 2019), we did not find clear evidence for that. For example, in the joint test, the lowest germination rate and the lowest share of TTC-positive tested seeds was observed with the seeds from A1-2012 which had the highest 100 kernel weight. In contrast, in the extended trial, the highest germination

rate and the highest share of TTC-positive tested seeds were observed with the youngest and heaviest seeds deriving from SEY. Thus, there was no clear correlation between seed weight and viability detectable. This is in accordance with Guillemin and Chauvel (2011) and Ortmans et al. (2016) who also concluded that seed weight had no influence on the seed viability of common ragweed. Similar, contradictory results were gained when observing other Asteraceae species like *Packera tomentosa* (Leverett & Jolls, 2014), implying that seed heteromorphism is not related to particular species, but very prominent within the Asteraceae-family and is influenced, not only by biotic and abiotic parameters like climatic and competitive conditions, but also by genetic parentage.

The same is true for the C/N-ratio which did not have any impact on the results. For example, the most viable seeds which were harvested in 2014 in SEY showed the highest N-percentage, but this did not differ significantly from the "weakest" population sampled in HAG in 2010. Viability studies on crops and different weedy species like *Amaranthus retroflexus* already showed that germination can be accelerated or decelerated by the N-content of seeds (Holdsworth et al. 2008; Karimmojeni et al. 2014). However, we could not find an influence of the initial N-content of common ragweed seeds on its ability to germinate.

Mean germination time

As with the germination rate, the MGT was strongly affected in both, the joint trial and the extended trial by the factor year, indicating that younger seeds germinate significantly faster than older seeds. However, even though the participating labs within the joint trial were using a standardised protocol, significantly different MGTs between labs were observed. To ensure the traceability of the results, the participants were provided with a blank form in the run-up to the joint-trial for submitting the results, but which also contained questions on storage and incubation conditions, as well as questions on the monitoring practice. Unfortunately, not all participants used this form for submitting the data. It is, therefore, quite difficult to understand these severe discrepancies in the MGT, even though - on request - all participants confirmed the correct application of the germination protocol.

With the extended trial, significant differences within years and between origins, respectively, were also observed, especially between the oldest seeds collected in the year 2010 which also showed the lowest germination rates. The amplitude of temperature-, moisture- and light conditions for germination following stratification is usually broad for common ragweed seeds in secondary dormancy (Baskin and Baskin 1998). Thus, the germination rate and the temporal distribution of germination can be strongly influenced by the temperature and light regime, especially when seeds were stored under controlled conditions (Baskin and Baskin 1980; Dinelli et al. 2013; Farooq et al. 2019). This underlines the importance of subsequent viability tests after the germination test which commonly takes 28 to 30 days and can often not be extended due to resource and time limitations.

Overestimation of viability in crush-test

The crush test is a widely accepted means of testing seed viability and various studies have shown that results of the crush test are comparable with other means like TTC testing (Sawma and Mohler 2002; Borza et al. 2007). Controversially, in the present study, the crush test led to a significant overestimation of viable seeds. On average, 55.6% more seeds were tested viable with the crush test when compared with both other testing methods. An explanation for this severe discrepancy could be the fact that seeds and embryos, respectively, of common ragweed are not only dead (no germination or discolouration in TTC) or empty (no embryo in the seed), but can also show indications of decomposition or degradation. When crushing these non-intact seeds, they also release moisture, which could be interpreted as liquid staining, hence leading to a misclassification as viable. Crush-test results indicate a false estimate of ragweed seed viability and should not be applied for scientific analyses.

Germination vs. TTC Test

In general, we can state that all germinated seeds can be classified viable, but some other viable seeds do not germinate due to seed dormancy. Viable seeds comprise of the germinated seeds plus the TTC-positive seeds anyway. This number of viable should be ± identical to the number of TTC-positive seeds. Even though testing viability by using TTC is common practice in broad fields of plant and microbiological research (AOSA 2000), significant differences between the results of the simple germination test and the TTC test were observed, particularly between the number of germinated seeds and the number of seeds tested positive and intermediate, respectively, with the TTC test. One possible reason for these discrepancies could be the categorisation of the state "intermediate" since this state covers a wide range of different colouration intensities (Fig. 9). The colouration might be addressed to differing viability stages (Karrer et al. 2016b). Thus, it is still unclear if these seeds are still viable or not and if there is a so-called threshold of colouration, below which seeds could be categorised as dead and beyond this, seeds would still be able to germinate. Since germination tests with these TTC-treated seeds are no longer feasible, statistical analysis was performed on the basis of the results of germination tests and TTC tests of the same seed cohorts, showing that the possibility of a TTC-intermediate tested seed to be viable was 92.87%, calculated over all populations and seed ages. Despite that high probability value, it was evident that, with increasing seed age, results were biased since for all intermediate tested seeds from the year 2010, a proper statistical classification was not possible, as the probability of the intermediate seeds to be viable or not was indifferent. Summarising, it could be assumed that the probability of a seed tested TTC-intermediate to be viable decreases with increasing seed age which is in accordance with numerous studies on seeds of different species (Bewley and Black 1982; Baskin and Baskin 1998; Walters et al. 2005; Harrison et al. 2007).

Figure 9. Examples of microscope pictures of seeds after TTC colouration classified by the joint-trial participants in the post-experiment evaluation with different accurrancy.

Differences between labs

With the joint trial, severe discrepancies in the evaluation of TTC-stained seeds were detectable, especially amongst labs, but not within labs when two independent testers were employed. However, even though the participants were provided with a standardised protocol giving information on testing procedures, evaluation practice, as well as storage and incubation conditions, not all participating labs followed these specifications. For example, in the Romanian lab, seeds were only incubated in TTC solution for 12 hours instead of the proposed 24 hours in the protocol. This could explain why both testers of the Romanian lab counted the significantly lowest number of fully-coloured (TTC-positive) seeds, whilst their share of TTC-intermediate coloured seeds was highest amongst all labs.

Another reason for these differences in the results could be a certain degree of uncertainty, particularly with the TTC-state "intermediate" (Figs. 1 and 9). For a proper classification of the seeds, the embryos have to be pulled out of the involucrum and the achene and have to be checked under a microscope since colouration and non-colouration, respectively, are often discreet and, therefore, not visible to the naked eye. If this is not done, the risk of classifying a seed as "positive" or "negative" instead of intermediate is quite high, as was the case in the Serbian lab. Over all populations, only 0.5% of the seeds were classified as intermediate due to improper evaluation practice.

Generally, it should be noticed that classification of TTC-stained seeds is, to some extent, due to subjectivity since the three different states are not always clearly divisible (Karrer et al. 2016b). To check the impact of subjectivity, subsequently to the joint trial, 50 microscopic pictures of TTC-stained seeds were sent to the participants of the joint trial who were ask to evaluate whether the seeds are positive, intermediate or negative. Results (Fig. 9) showed clearly that subjectivity has an impact to a very large extent. Only with 17 out of 50 seeds, a unanimous result was given. With 12 seeds, all states (positive, intermediate and negative) were represented and the remaining 31 seeds were either classified as positive or intermediate and intermediate or negative, respectively (Fig. 9).

Conclusions

Viability of common ragweed seeds is strongly influenced by age and origin. However, various environmental factors (light conditions, temperature, nutrient availability, soil type etc.), as well as storage conditions, have to be considered when testing for viability of seeds. Particularly with joint trials, this study clearly reveals the problems involved in such ring-experiments. On the one hand, some results were not traceable even though participants were provided with standardised protocols and forms. On the other hand, subjectivity in evaluating results led to significant discrepancies amongst labs. As a consequence, the aim is to develop improved protocols and evaluation standards, especially for TTC testing to ensure that future joint tests show better comparability and traceability of results. This study, therefore, contributes to the improvement of testing standards for estimating the infestation rate of any containment with common ragweed. Germination tests plus the subsequent TTC tests of the remaining seeds gave almost the same number of viable seeds anyway and is, therefore, the most reliable testing method. Thus, we can recommend both test strategies because of ± equal validity. If time is short, the stand-alone TTC test achieves sufficient validity if subjectivity in colours' interpretation is reduced. The crush test only gives not really valid estimates of viable common ragweed seeds. Furthermore, we would like to underline that the testing labs should strictly follow the actualised guidelines (i.e. Karrer et al. 2016c).

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Supplementary material 1

Supporting tables and figures

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Data type: tables and figures

Explanation note: **Table S1.** Results of the ANOVA showing significant differences among labs (incl. tester-code) with the results of the stand-alone TTC-test on ragweed seed viability of Austrian seed lots (percentages of TTC-positive, TTC-intermediate and TTC-negative per population); different letters indicate significant differences between the seed lots within the respecive staining class. **Figure S1.** Germination rate [%] of ragweed seeds in relation to the factors origin and age (n = 1,536; different letters indicate significant differences between origins; codes see in Tab 4). **Table S2.** Results of Chi³-Test: Probability that randomized samples for germination test plus subsequent TTC-test (Col. 1) and stand-alone TTC-test (Col.2) as well as all samples taken (Col. 3) originate from same overall populations (n = 7,200); as indicator values the number of germinated and/or TTC-positive tested seeds were used.

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