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Seed germination behavior of two *Brachypodium* species with a key role in the improvement of marginal areas

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Abstract

Brachypodium genuense (DC.) Roem. & Schult. and *B. rupestre* (Host) R. et S. are important components of the vegetation of some widespread secondary, semi-natural grassland habitats. Both species play a very important role in the development of vegetation series that characterize these grasslands when they are no longer subjected to grazing or cutting regimes. This led to the gradual disappearance of such habitats and the constitution of new woods.

In some cases, such as roadsides and marginal areas, it could be convenient to facilitate this serial process by seeding or hydroseeding of native species of the genus *Brachypodium*. This approach could led to a better evolution of the soil with the reduction of erosion, the constitution of more natural woods and the reduction of fires risk. For this reason the germplasm of a population of *Brachypodium genuense* and four populations of *B. rupestre* from Central Apennines was collected and its germination behavior was studied.

Indeed, the early phases of seedling development are critical to the successful establishment of grassland species. Precisely, it was investigated the influence of the following factors on germination: seed size and weight, temperature, light and the removal of outer covering structures. Indeed, each of the above-mentioned factors affects technical aspects of the sowing. Inter- and intra-species variations in seed germination behavior were evidenced in this work. Light was found to enhance germination in both species, whereas remarkable differences have been found in temperature requirements between the two species and also among the four populations of *B. rupestre*.

Keywords: *Brachypodium genuense*, *Brachypodium rupestre*, ecology, grassland, marginal areas, seed germination, semi-natural grassland, vegetation series, habitat restoration.

Introduction

The study of semi-natural Apennine grasslands has been the object of numerous researches since they are habitat especially endangered after the loss of economic interest that determined their abandonment (Baldoni *et al.*, 2004; Ballerini & Biondi, 2002; Biondi *et al.*, 2000; Catorci *et al.*, 2012; Catorci *et al.*, 2011a).

The abandonment of agricultural and pastoral activities concerning their management has determined the start of spontaneous serial processes of vegetation recovery which caused the recovery of potential bush and wood flora in wide areas of mountain and hilly areas but also the disappearance of very important environments in terms of phytocoenotic, floristic and, more in general, ecological biodiversity (Biondi *et al.*, 2006, 2009, 2012a).

The disappearance or strong reduction of this habitat affects trophic chains and determines a remarkable loss of biodiversity at every level, causing a simplification of the landscape. Therefore, the Habitats Directive (92/43/EEC) considers semi-natural grasslands conservation very important and possibly their recovery in terms of specific and habitat biodiversity. This led to the census of these habitats in the EU and to the definition of management plans of the sites of interest which are currently ongoing in all the Natura 2000 Network.

But, there is a high degree of biodiversity also in agro-ecosystems, therefore new policies in agriculture (CAP) promote the change of traditional farmlands into high nature value rural areas where productivity is strictly linked to conservation of biodiversity (Bignal & McCracken, 2000; Andersen *et al.*, 2003; Galdenzi *et al.*, 2012: Paracchini *et al.*, 2008).

The practices that are usually used are the reinstatement of active management and also the removal of the shrubs that invaded the grassland. But sometimes, there are some technical problems which are difficult to overcome. The first is linked to the serial recovery process of vegetation started by species of the genus *Brachypodium*, which do not whet animals appetite due to the consistency of their leaves that are rich in silica and lignin (Catorci *et al.*, 2013; Roggero *et al.*, 2002) and long rough hairs. Moreover, animals risk to die if they are obliged to feed with these plants (Scocco *et al.*, 2007; 2012). It is well documented that *Brachypodium* sp.pl. reduces or stops the natural dynamic processes in the evolution of grasslands towards more mature stages of vegetation series (Bonanomi & Allegrezza, 2004; Bonanomi *et al.*, 2006, 2009; Catorci *et al.*, 2011a; Hurst & John, 1999; Endresz *et al.*, 2005). The other problem is linked to the difficulty to remove shrubs since it needs to be followed by a seeding of herbaceous species. Unfortunately, commercial seed

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mixtures contain species with an extra European origin and autochthonous seeds are not available. Thus, the use of such seed mixtures determines a heavy erosion of biodiversity.

For all these reasons, the research team, that the authors belong to, started studies and projects on this subject, such as the research here presented.

This research focused on seed germination requirements since the early phases of seedling development are critical to successful establishment of grassland species and since germination and emergence are important parameters that determine the potential population of individual species in restored environment (Lonati *et al*., 2009). More precisely, this research studied the genetic and environmental factors affecting germination of seed of *Brachypodium genuense* and *B. rupestre*, with particular attention to their technical repercussions.

*Brachypodium genuens*e and *B. rupestre* are two different species of the same genus occurring in central Apennines. Both have features of dominant species characterizing by large dimensions, strong capacity of vegetative reproduction, growth from basal meristems and high phytomass production (Lucchese, 1987; Camiz *et al*., 1991). Because of these features, these species spread and start to increase their dominance in the abandoned conditions until becoming invasive and altering the ecological status of the site (Bonanomi & Allegrezza, 2004; Bonanomi *et al*., 2006, 2009; Catorci *et al*., 2011b).

Nevertheless, they show a quite different ecology regarding particularly soil preference and distribution along the altitudinal gradient (Dowgiallo & Lucchese, 1991). *Brachypodium rupestre* is a pioneer species, growing on poor basic soils mostly deriving from calcareous rocks, even if it also occurs on clays. *Brachypodium genuense* occurs at higher altitudes (from montane to high montane belt) on deep and rich soils deriving from sandstones but having an acid-subacid reaction (pH from 4.5 to 7.0).

As regards the morphological and histologic differences, the two species belong to different life forms: *B.*
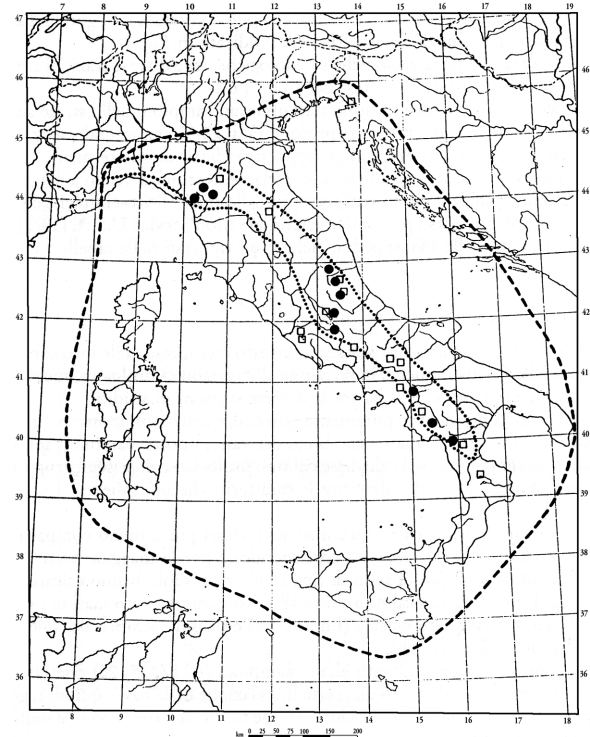

Fig. 1 - Range of distribution of *Brachypodium rupestre* (---) and *B. genuense* (....) in Italy. From Dowgiallo and Lucchese (1991) redrawn.

rupestre is a rhizomatous hemicryptophyte while *B. genuense* is a caespitose hemycriptophyte. Furthermore, there are differences in the leaves shape and anatomy and in the morphology of spikelets (Lucchese, 1988).

The Italian distribution of the two species mostly overlaps; *B. rupestre* having a wider range of distribution that contains the distribution range of *B. genuense* focused in the Apennine chain (fig. 1).

Materials and methods

Seed collection

The germination behavior of four populations of *Brachypodium rupestre* and of a population of *B. genuense* was studied. Mature seeds were collected from wild

Tab 1 - Collection sites with GPS coordinates of each population studied.

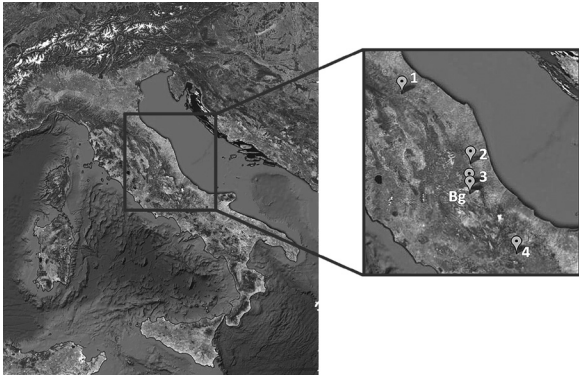| SPECIES	POPULATION	SITE OF COLLECTION		
		LOCALITY	ALTITUDE (masl)	WGS84
Brachypodium genuense	1	Gran Sasso e Monti della Laga N. Park - Campo Imperatore (AQ)	1582	42.393007°	13.563239°
Brachypodium rupestre	1	Sasso Simone e Simoncello Park -Pian dei Prati (PU)	733	43.758307°	12.350129°
	2	Gran Sasso e Monti della Laga N. Park -San Giacomo (TE)	1042	42.807141°	13.573715°
	3	Gran Sasso e Monti della Laga N. Park - Prati di Tivo (TE)	1472	42.497711°	13.555683°
	4	Macchiagodena (IS) 2	947	41.565544°	14.393311°

Fig. 2 - Map of collection sites of *B. genuense* (Bg) and *B. rupestre* (1, 2, 3 and 4).

populations during summer 2010 (*B. rupestre*) and summer 2011 (*B. genuense*) following international protocols (ISTA 2004, 2006; Bacchetta *et al.*, 2006).

Following harvest, seeds were cleaned by gently grinding the spikelets on a rubber mat and samples processed to remove empty and poorly developed seeds with a blower (Agriculex CB1 Column Seed Cleaner, T.A. Baxall and Co., Ltd). Afterwards, they were dried and stored in a dry room at 15°C and 15% relative humidity for 3 to 6 months before being used for germination testing and morphological analysis.

Seeds were collected in different areas of Central Apennines (Fig.2; Tab.1,). Climatic data for each site of collection were obtained from World Clim database (Hijmans *et al.*, 2005; not reported here).

Morphological analysis

Length, width and thickness of twenty caryopses (palea and lemma removed) for each seed lot were measured with calipers and a Nikon C-PS SMZ645 stereoscope, fitted with a C-W10X/22 micrometer (Southern Microscopes, Maidstone, UK). Four samples of ten seeds of each seed lot were weighed on a seven-place balance (Mettler Toledo UMT2, Beaumont Leys, UK) with a precision of 0.1 μg. X-ray analysis were carried out on a sample of 50 seeds for each population to detect empty, poorly developed or damaged seeds. A Faxitron digital X-ray machine (Qados, Sandhurst, UK) set at the standard Millennium Seed Bank settings for seed X-ray radiography (22kV and 0.3 mA for 20 s) was used. Samples were randomly selected.

Germination tests

Seeds were sown on 1% distilled water agar held in 9 cm diameter transparent polyethylene Petri dishes. Germination response was tested in programmable-environmental chambers with controlled temperature and illumination. Germination response to temperature was evaluated at 7 constant temperatures ranging between 5 and 35°C. Illumination was provided for 12 hours each day by 30 W cool white fluorescent lights.

For dark treatments (at 20°C only), Petri dishes were wrapped in two layers of aluminum foil.

Seeds of a population of *B. rupestre* were tested with their covering structures (palea and lemma) intact and with these structures removed.

Four replicates of 25 seeds each were used in each germination test. The seeds were monitored daily until germination ceased, then they were monitored progressively less frequently, for at least 30 days after sowing. Germinated seeds were removed when radicle was at least 1 mm long (Bacchetta *et al.*, 2006). For tests in the darkness of *B. rupestre* population1 and of *B. genuense*, germination was scored with the same frequency of tests in the light in a dark room under a dim safe, green light comprising three 15-20 W cool white fluorescent tubes covered by three layers of no. 39 (primary green) Cinemoid as described in Probert and Smith (1986). The seeds of the other three populations of *B. rupestre* tested in the darkness were scored just at the end of the tests, after 30 days from sowing.

Germination tests were considered finished when no additional seeds germinated over a period of at least 15 days.

At the end of each germination test, seeds which had not germinated were dissected (cut-test) to determine whether they were viable (fresh), non-viable (mouldy) or empty.

Data analysis

Seed volume was calculated with the following equation:

$$VOL = \pi LWT/6$$

where VOL is seed volume, L is length, W is width and T is thickness. (Casco & Dias, 2008).

Seed volume and weight mean ± standard deviation were calculated for each population.

As seed volume and weight data did not show homogeneity of variance, a non-parametric test was used to tests for significant differences in seed volume and weight between populations of the same species at the 5% level. A Mann-Whitney U test was used when there were only two populations to compare, a Kruskal-Wallis One Way ANOVA was used for the other species.

All analyses were carried out using GenStat release 15.1 (VSN International Ltd., UK).

The FITNONLINEAR directive with a probit link function and binomial error distribution was used to fit the equation,

$$g = \Phi(\beta_0 - \beta_1(p(T - T_{base}))^{-1})$$

to the germination progress data (period from sowing, cumulative number of seeds germinated) at sub-optimal temperatures. In this equation, g is germi-

Tab. 2 - Mean and standard deviation values for length (n=20), width (n=20), thickness (n=20), volume (n=20) and weight (n=4x10) of seeds of the population of *B. genuense* and four of *B. rupestre*.

SPECIES	POPULATION	Mean Length	STDV Length	Mean Width	STDV Width	Mean Thickness	STDV Thickness	Mean Volume	STDV Volume	Mean Weight	STDV Weight
		(mm)	(mm)	(mm)	(mm)	(mm)	(mm)	(mm3)	(mm3)	(mg)	(mg)
Brachypodium genuense	1	6.340	0.5368	1.402	0.1649	0.856	0.1142	4.035	1.566	4.161	0.2079
	1	6.360	0.6898	1.232	0.1287	0.641	0.0777	2.671	0.6891	4.036	0.2264
	2	6.382	0.5424	1.365	0.1233	0.675	0.0815	3.066	0.4361	3.664	0.1581
	3	6.243	0.5658	1.430	0.1354	0.634	0.0832	2.971	0.6013	3.681	0.2342
Brachypodium rupestre	4	6.692	0.4159	1.388	0.1379	0.654	0.1132	3.167	0.5828	3.823	0.1443

nation (proportion of seeds sown), Φ is the cumulative normal distribution function, β_0 is the maximum germination in probits and β_1, the thermaltime constant (θ_T), describes the rate of reduction in probit germination as the reciprocal of thermaltime above T_{base} (base temperature) increases. In this analysis, the parameters β_0, β_1 and T_{base} were estimated concurrently. The sub-optimal temperature range was taken as 5°C up to and including the temperature where maximum % germination was observed.

The FITNONLINEAR directive was also used to fit split-line regression models to the germination rate data p_g^{-1} for proportion of sown seeds that germinated, $g = 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9$, versus temperature. Independent split-line regressions for $g = 0.1, 0.2, .., 0.9$ were fitted to identify the values of g for which data could be included (i.e. where there was sufficient data either side of a T_{opt}). The T_{base} and ceiling temperature, $T_{ceiling}$ are the temperatures where $p_g^{-1} = 0$ for the sub- and supra-optimal temperature ranges, respectively; the optimum temperature, T_{opt} is the break-point of the split-line regression. The p_g^{-1} data were calculated from the raw cumulative germination data as the inverse of the period of time from sowing needed to reach a proportion of germinated seeds, g.

Where split-line regression analysis was not possible for a seed lot, T_{base} was estimated through linear regression analysis of p_g^{-1} versus temperature, for the sub-optimal range.

Mean germination percentage and mean germination time (MGT) were calculated for tests in the light and

in the dark and for tests on seeds with or without their covering structures. Mean germination time (MGT) was calculated according to the equation of Ellis and Roberts (1980):

$$MGT = (n\ d)/N$$

where n is the number of seeds which germinate on day d, and N is the total number of seeds germinated at the end of the test. A logistic regression analysis was used to find significant differences on germination response to light and to the removal of outer covering structures.

All analyses were carried out using GenStat release 15.1 (VSN International Ltd., UK).

Results and discussion

Morphological analysis

As regards *B. rupestre*, seeds from population 1 had the lowest mean volume but the highest mean weight (Tab. 2). However, no statistical difference was found in mean seed volume with Kruskal-Wallis one-way ANOVA (P = 0.077), while the test could not be performed on mean seed weight values. *B. genuense* seeds were found to have higher volume and weight compared to *B. rupestre* seeds. No empty or poorly developed seeds were detected in the samples which were x-ray analysed.

Germination tests

Brachypodium genuense seeds germinated to between 14% (at 5°C) and 87% (at 25°C) (Tab. 3). Final germination values obtained at 25 and 30°C were considerably higher than those obtained at the other temperatures tested. The seeds showed a large delay in the start of germination at 5°C, indeed germination started 54 days after sowing (Fig. 3). The speed of germination increased with temperature between 5 and 25°C, with the exception of T_{10}, and decreased between 25 and 35°C (Fig. 4). Fitting a thermal time model to the data for sub-optimal temperatures, the estimated T_{base} was 8.5 ± s.e. 0.33°C (Fig. 3). However, this model did not seem to fit the data of this species properly. This is

Tab. 3 - Final germination percentages at temperatures between 5 and 35°C for seeds from a population of *B. genuense* and four populations of *B. rupestre*.

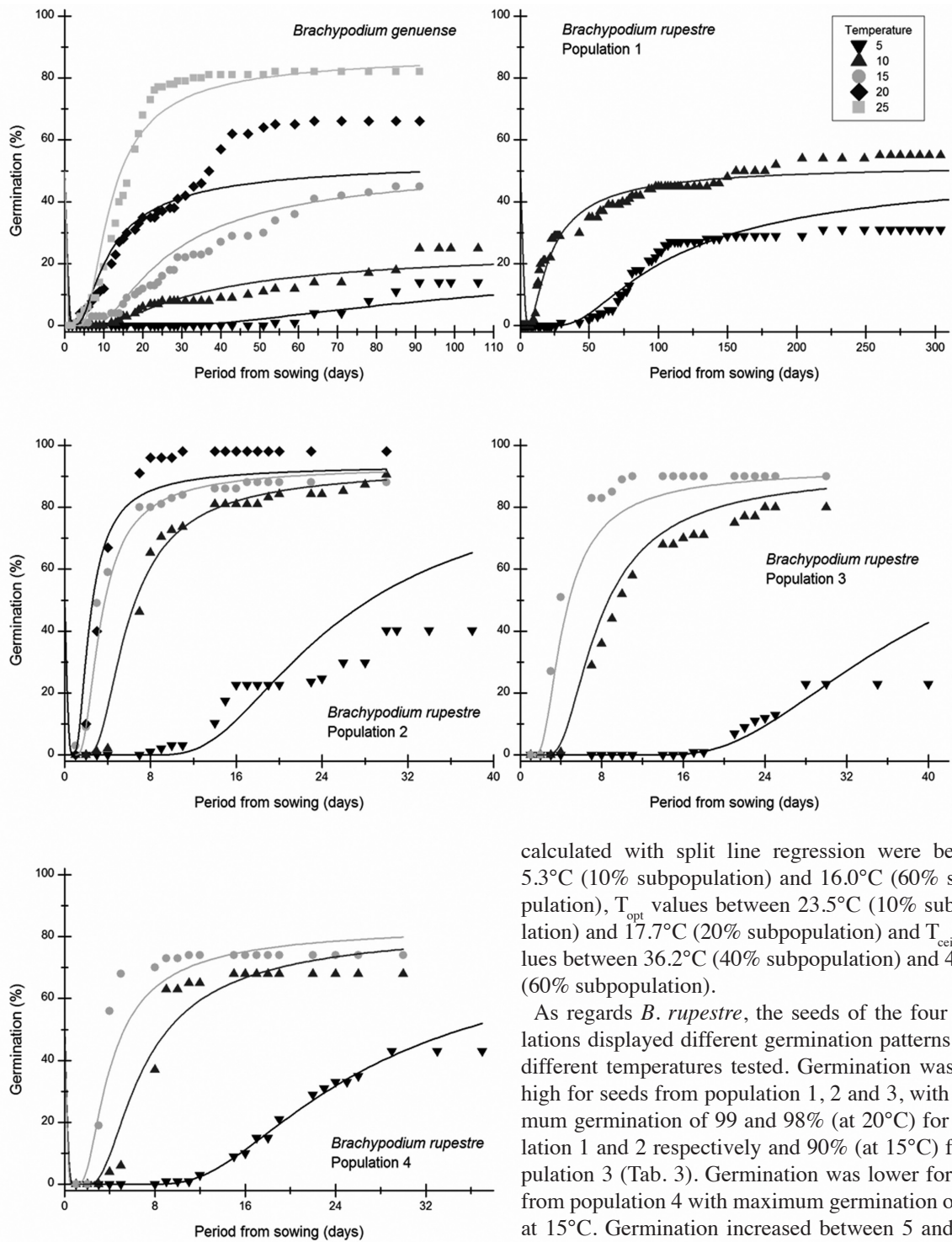| SPECIES	POPULATION	FINAL GERMINATION (%)						
		5°C	10°C	15°C	20°C	25°C	30°C	35°C
Brachypodium genuense	1	14	25	47	69	87	86	22
	1	31	55	87	99	94	80	13
	2	40	91	88	98	95	89	49
	3	23	80	90	83	72	54	12
Brachypodium rupestre	4	43	68	74	60	52	43	18

Fig. 3 - Results of fitting the thermal time model to cumulative germination percentage at sub-optimal temperatures for seeds from a population of *B. genuense* and four populations of *B. rupestre* tested at temperatures between 5 and 35°C.

probably due to the large differences in final germination obtained at suboptimal temperatures. Independent split line regression of the p_g^{-1} data was possible for subpopulations between 10 and 60%. T_{base} values

calculated with split line regression were between 5.3°C (10% subpopulation) and 16.0°C (60% subpopulation), T_{opt} values between 23.5°C (10% subpopulation) and 17.7°C (20% subpopulation) and $T_{ceiling}$ values between 36.2°C (40% subpopulation) and 40.6°C (60% subpopulation).

As regards *B. rupestre*, the seeds of the four populations displayed different germination patterns at the different temperatures tested. Germination was quite high for seeds from population 1, 2 and 3, with maximum germination of 99 and 98% (at 20°C) for population 1 and 2 respectively and 90% (at 15°C) for population 3 (Tab. 3). Germination was lower for seeds from population 4 with maximum germination of 74% at 15°C. Germination increased between 5 and 20°C and decreased between 20 and 35°C in seeds from populations 1 and 2. It increased between 5 and 15°C and decreased between 15 and 35°C in seeds from populations 3 and 4. Seeds from all the populations tested showed a delay in the start of germination at 5°C; germination started after 30, 7, 16 or 9 days for seeds from population 1, 2, 3 and 4, respectively (Fig. 3). The speed of germination responded differently to temperature in the seeds of the populations studied and sometimes also in subpopulations of data (Fig. 4).

Tab. 4 - Cardinal temperatures values estimated with Split-Line Regression Model from data of the 50% subpopulation and T_{base} estimated with Fitnonlinear Model for seeds from a population of *B. genuense* and four populations of *B. rupestre*.

SPECIES	POPULATION	SPLIT LINE REGRESSION MODEL			FITNONLINEAR MODEL
		T_{base}	T_{opt}	$T_{cieling}$	T_{base}
		(°C)	(°C)	(°C)	(°C)
Brachypodium genuense	1	15.9	26.9	36.4	8.5
	1	9.4	23.9	47.3	9.7
	2	5.6	17.6	35.1	3.4
	3	N/A	N/A	N/A	3.7
Brachypodium rupestre	4	N/A	N/A	N/A	3.2

Tab 5 - Mean germination percentage, MGT and P value calculated with Logistic Regression for tests in light and dark at 20°C in seeds from a population of *B. genuense* and four populations of *B. rupestre*.

SPECIES	POPULATION	ILLUMIN. REGIME	GERMINATION	P	MGT
			(%)		(days)
Brachypodium genuense	1	light	68.4	<0.001	23.75
		dark	31.2		26.15
Brachypodium rupestre	1	light	99.0	0.006	5.06
		dark	90.9		5.45
	2	light	98.0	<0.001	
		dark	64.0		
	3	light	83.0	0.001	
		dark	63.0		
	4	light	60.0	0.040	
		dark	73.9		

Fitting a thermal time model to the data for sub-optimal temperatures, the estimated T_{base} was very similar in seeds from populations 2, 3 and 4 (3.5 ± s.e. 0.12°C, 3.8 ± s.e. 0.08°C and 3.2 ± s.e. 0.13°C, respectively) and considerably higher in seeds from population 1 (9.7 ± s.e. 0.18°C). The speed of germination was maximum at 25°C in seeds from population 1 and 2, at 30°C in seeds from population 4 and it varied differently according to the subpopulations of data in population 3. It was not possible to perform a split-line regression on population 4 data, since the speed of germination was maximum at 30°C and germination drastically decreased at 35°C, so that the only datum available at this temperature was the T_{10}. As well, the model did not fit the data of all the subpopulations and, in the cases of seeds from populations 1 and 3, the model failed to estimate the $T_{ceiling}$ value or provided unrealistic estimates. The estimated T_{base} was between 4.1°C (20% subpopulation) and 9.8°C (80% subpopulation) for seeds from population 1, between 2.9°C (10% subpopulation) and 5.4°C (40% subpopulation) for population 2, between 3.8°C (20% subpopulation) and 5.1°C (60% subpopulation) for population 3 and

between 1.9°C (30% subpopulation) and 3.3°C (40% subpopulation) for population 4. The estimated T_{opt} was between 23.9°C (50% subpopulation) and 29.5°C (10% subpopulation) for seeds from population 1, between 26.3°C (10% subpopulation) and 28.9°C (40% subpopulation) for population 2, between 16.2°C (20% subpopulation) and 18.23°C (60% subpopulation) for population 3 while it was not possible to estimate this value for seeds from population 4. The estimated $T_{ceiling}$ (excluding not realistic data) was between 31.4°C (80% subpopulation) and 42.2°C (60% subpopulation) for seeds from population 1, between 36.9°C (40% subpopulation) and 40.8°C (10% subpopulation) for population 2 and between 29.4°C (70 and 80% subpopulations) and 39.2°C (40% subpopulation) for population 3. As regards the influence of light on germination, logistic regression showed that light clearly increased germination in seeds of *Brachypodium genuense* (P < 0.001), whereas darkness increased the speed of germination (Tab. 5). Similarly, light clearly increased germination in seeds from populations 1, 2 and 3 of *B. rupestre*, whereas it decreased germination in seeds from population 4. Logistic regression analysis showed that all the differences were significant. Germination was slightly slower in the darkness.

The effect of outer covering structures (palea and lemma) was investigated only on seeds from population 1 of *B. rupestre* and the removal of such structures was found to be of no effect. Indeed, seeds germinated equally well either intact and with palea and lemma removed (Tab. 6). Naked seeds just had a higher speed of germination.

Discussion

Seed Size and Weight and their relationship with germination performance

The morphological analyses conducted on *Brachypodium rupestre* could not detect any differences in seed size in the four populations studied (Tab. 2). Conversely, there seems to be quite a clear difference in seed weight values, at least among seeds from population 1 and the others. No clear relationship between seed weight and volume were found in this species, indeed, seeds from population 1 showed the highest weight and the lowest volume.

Seed weight values were compared with other published values of air-dry seeds of the same species: 2.57 mg (Cerabolini *et al.*, 2003) for *B. rupestre* and 1.95 mg for *B. rupestre* ssp. *caespitosum* (Piccinin *et al.*, 2004). Both values are lower than the values of seeds from the four populations studied (between 3.68 and 4.04 mg). However, seed weight and volume did not seem to affect germination performance in this species, neither in terms of mean germination, nor in terms of maximum germination.
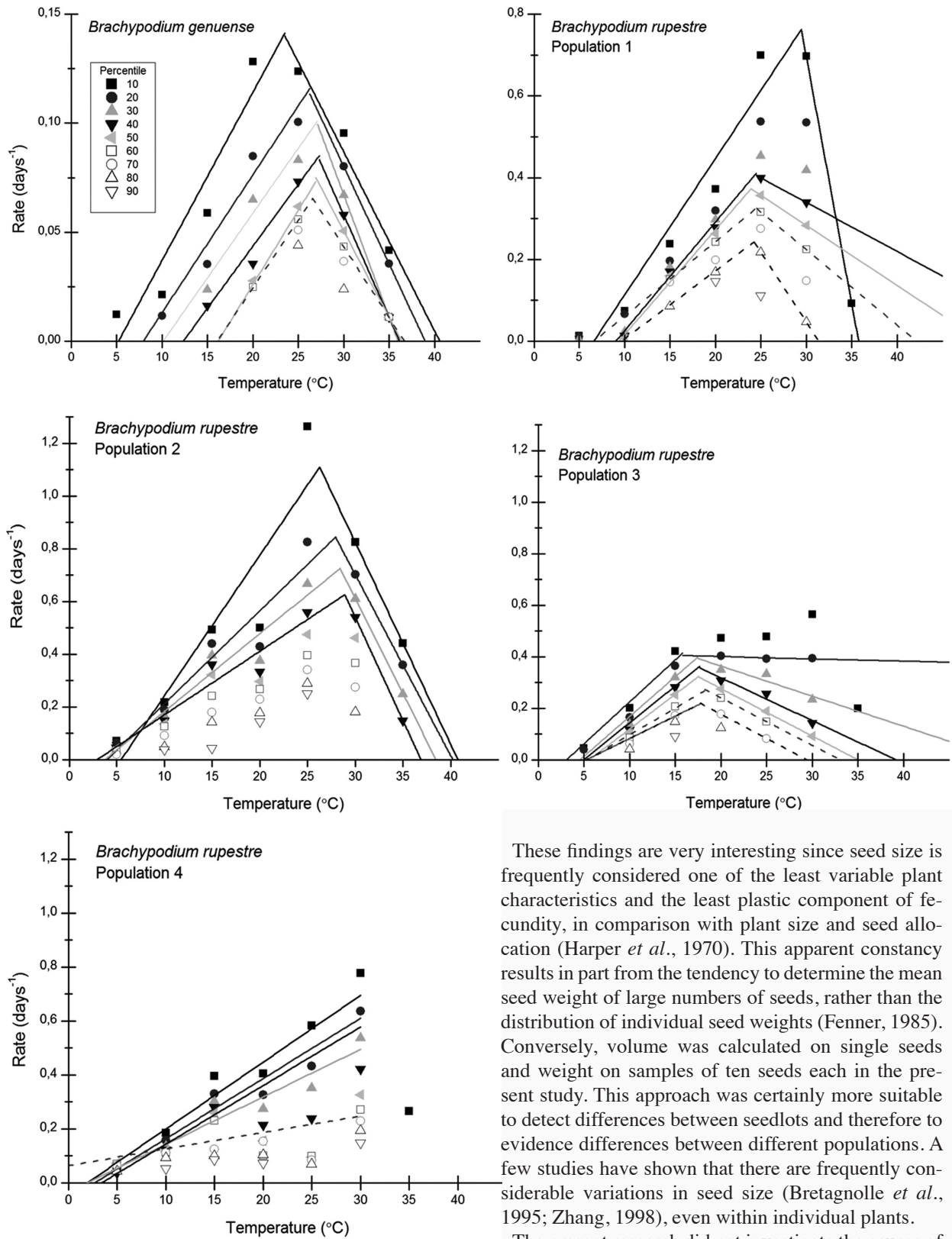
Fig. 4 - Split-line regression model p$_\mathrm{g}^{-1}$ for seeds from a population of *B. genuense* and four populations of *B. rupestre* at temperatures between 5 and 35°C.

These findings are very interesting since seed size is frequently considered one of the least variable plant characteristics and the least plastic component of fecundity, in comparison with plant size and seed allocation (Harper *et al.*, 1970). This apparent constancy results in part from the tendency to determine the mean seed weight of large numbers of seeds, rather than the distribution of individual seed weights (Fenner, 1985). Conversely, volume was calculated on single seeds and weight on samples of ten seeds each in the present study. This approach was certainly more suitable to detect differences between seedlots and therefore to evidence differences between different populations. A few studies have shown that there are frequently considerable variations in seed size (Bretagnolle *et al.*, 1995; Zhang, 1998), even within individual plants.

The present research did not investigate the causes of such differences, so it is not possible to establish whether they are due to genetic factors or not. Although in this research seed weight and volume values and climatic data of collection sites (not reported here) have

Tab. 6 - Mean germination percentage, MGT and P value calculated with Logistic Regression for tests on intact and with palea and lemma removed seeds from population 1 of *Brachypodium rupestre* at 20°C.

SPECIES	POPULATION	Seeds	Germination (%)	P	MGT days
Brachypodium rupestre	1	with palea and lemma removed	100.0		3.36
		intact	99.0	0.236	5.060.417

been studied for each population, it is not possible to establish whether a relationship exists between such data. That is because the amount of populations studied for each species is too low for this purpose.

Moreover, it has been demonstrated that variations not only pertain to seed size but also colors and shape of seeds (Baskin & Baskin, 1998). Such variations are due to both genetic and environmental factors during the time of seed development. Among the environmental factors producing the previously mentioned variations, there is mineral nutrition; precisely, high levels of nitrogen (Gibson & Humphreys, 1973), phosphorus (Lewis & Koide, 1990), potassium (Willson & Price, 1980; Parrish & Bazzaz, 1985) or mixed mineral nutrients (van Andel & Vera, 1977) in the soil increase seed size in some species. Other factors found to affect seed size, either increasing or decreasing it, are: soil moisture (Schimpf, 1977; Brocklehurst *et al.*, 1978; Withers & Forde, 1979; Chadoeuf-Hannel & Barralis, 1982; Meckel *et al.*, 1984; Ramseur *et al.*, 1984; Stamp, 1990 Stromberg and Patten, 1990), solar irradiance and day length (Williams, 1960; Williams & Harper, 1965; Cook, 1975; Brocklehurst *et al.*, 1978; Jenner, 1979; Martinez-Carrasco & Thorne, 1979; Willson & Price, 1980; Agren, 1989; Schmitt *et al.*, 1992; Sultan, 1996), temperature (Lambert & Linck, 1958; Stearns, 1960; Maun *et al.*, 1969; Wardlaw, 1970; Bean, 1971; Datta *et al.*, 1972; Skerman & Humphreys, 1973; Ford *et al.*, 1976; Akpan & Bean, 1977; Egli & Wardlaw, 1980; Wood *et al.*, 1980; Campbell *et al.*, 1981; Alexander & Wulff, 1985; Mohamed *et al.*, 1985; Wulff, 1986; Drew & Blocklehurst, 1990; Lacey, 1996), the timing in which seeds are produced during the growing season (Soffer & Smith, 1974; Raju & Ramaswamy, 1983; Cavers & Steel, 1984; Thompson & Pellmyr, 1989; Kane & Cavers, 1992) and the position; indeed, seeds produced in different parts of the same inflorescence may differ in weight (McGinley, 1989); this evidence has been found in a few grasses (Whalley *et al.*, 1966; Lambert, 1967; Datta et. al, 1970).

It was important to study the variation of seed size among different populations of the same species since it is considered as an important trait determining the successful establishment of individual plants (Westoby *et al.*, 1992; Vaughton & Ramsey, 1997; 1998; Zhang, 1998). Indeed, seed mass represents the amount of

maternal investment for individual offspring. Generally, seed weight variation is associated with fitness and population establishment since seed traits are critical elements in the life history of plants. In agronomic species, seed weight is correlated with seed vigor, plant growth, and even yield (Lafond & Baker, 1986; Berdahl & Frank, 1998; Boe, 2003). Seed weight has been found to have a positive effect on germination percentage in a large number of species, either in laboratory (Thompson, 1990; Bretagnolle, 1995) or in field conditions (Roach, 1987; Winn, 1988).

In wild plants, large seed size is correlated with a higher seedling recruitment (Negri & Falcinelli, 1990; Mendez, 1997; Susko & Lovett-Doust, 2000; Dalling & Hubbell, 2002; Debain *et al.*, 2003), bigger seedlings (Hou & Romo, 1998) and greater probability of survival (Simons & Johnston, 2000; Walters & Reich, 2000). Ecologically, seedlings emerging from large seeds often survive longer than those from small seeds under adverse seedbed conditions, such as low light (Simons & Johnston, 2000), low water (Hendrix & Trapp, 1992; Chacon & Bustamante, 2001), nutrient limitations (Vaughton & Ramsey, 1998) and deep burial depth (Yanful & Maun, 1996; Ruiz-de-Clavijo, 2002).

Germination requirements and behavior
TEMPERATURE

Brachypodium genuense seeds germinated best at high temperatures (25-30°C). Moreover, since the maximum germination percentage was only 87%, perhaps some seeds were still dormant when germination tests were conducted. A remarkable number of seeds failed to germinate at temperatures between 5 and 20°C. Moreover, germination started with a very long delay (54 days) at 5°C. For all these reasons, cardinal temperatures values were considerably high in this species. These are very meaningful findings, in ecological terms, because this species grows at a higher altitude, compared to the other studied here, and thus experiences the lowest temperatures (mean, minimum and maximum annual temperatures). Seeds germination behavior appears to be strongly influenced by the environment, as it was reported for species other than *Poaceae* (Sawhney & Naylor, 1979; Probert *et al.*, 1985a; Simpson, 1990). Such behavior could reflect a survival strategy aimed to avoid early germination in a period where extreme cold events are likely to occur

(Derkx, 2000). For all this reasons and since the level of innate dormancy in seeds usually declines during dry storage (Probert, 1992), it would have been particularly interesting to study germination of freshly-harvested seeds in this species, in order to establish whether they have dormancy or not, how deep it is and how to break it. In fact, physiological dormancy is quite common in *Poaceae* (Simpson, 1990; Baskin & Baskin, 1998). Furthermore, different *Brachypodium genotypes* display dormancy (Barrero *et al.*, 2012) and a physiological dormancy has been found in seeds of *B. sylvaticum* (Grime *et al.*, 1981) and *B. distachyon* (Barrero *et al.*, 2012).

As regards *B. rupestre*, germination performance varied quite noticeably among the different populations tested (Tab. 3). The highest mean germination was found in seeds from population 2 while the highest maximum germination in seeds from population 1. The lowest germination was observed in seeds from population 4.

The germination rate varied among the populations within the same temperature and in some cases also for subpopulations data within the same population (Fig. 4). Similarly, the delay in the start of germination at 5°C was very different among populations (7-30 days) (Fig. 3).

Conversely, T_{base} values estimated with the thermal time model were very similar in seeds from populations 2, 3 and 4 (3.5, 3.7 and 3.2°C, respectively). Independent split-line model does not seem to properly fit the data. The only data available for the 50% subpopulation refer to seeds from populations 1 and 2. The difference between these parameters and the parameters estimated using the thermal time model were 0.32 and 1.84°C for population 1 and 2, respectively, thus the estimates obtained with the two models were quite similar.

It is interesting to note that seeds from population 3, whose site of collection has a far higher altitude and consequently the lowest temperatures (mean, minimum and maximum annual temperature) (Tab.1), had the lowest cardinal temperatures, estimated with the split-line regression on the 50% subpopulation data.

It is interesting to note that two populations of *B. rupestre* reached the highest germination at 20°C and the other two at 15°C. T_{base} values estimated with Fitnonlinear Model were very similar in three out four populations of *B. rupestre*. T_{base} values estimated with both models were considerably different in *B. genuense*.

It was of paramount importance to study germination response to temperature since it is the single most important factor regulating germination of non-dormant seeds in irrigated, annual agroecosystem at the beginning of the growth season where light, nutrients and moisture are typically not growth limiting (Garcia-Huidobro *et al.*, 1982). It has a direct control on the rate of many chemical reactions, including respiration and photosynthesis (Munir *et al.*, 2004). Roberts (1988) recognized three separate physiological processes in seeds affected by temperature: first, temperature, together with moisture content, determines the rate of deterioration in all seeds; secondly, temperature affects the rate of dormancy loss in dry seeds and the pattern of dormancy change in moist seeds; and, thirdly, in non-dormant seeds temperature determines the rate of germination. Although a relationship between cardinal temperatures for each population studied and the climate of their own collection sites was not found, probably due to the low amount of data, further studies should be needed to verify this hypothesis that has been demonstrated for other species. Indeed, Probert (2000) and Baskin & Baskin (2001) found that germination response to temperature is related to ecological and geographical distribution of species and ecotypes, because germination is a critical stage of the life cycle reflecting adaptation to local habitats (Gutterman, 2000; Probert, 2000).

Based on studies with nematodes, Trudgill & Perry (1994) suggested that the temperature responses of poikilothermic species reflected the environments to which they were adapted and that differences between species have considerable ecological significance. It has been demonstrated that seeds of many grasses found in habitats characterized by summer drought, like the grasses of this study, are capable of germination under a wide range of temperatures, although timing of germination is determined by the amount of moisture (Thompson & Grime, 1979).

The results of germination tests suggest that seeds of the grasses studied probably start germinating during autumn when temperatures are above the estimated T_{base} values and soil moisture is not limiting. Germination of these species continues through the winter until cold soil limits germination; germination begins again in spring when soil temperatures warm and soil moisture remains not limiting. For all these reasons, sowing should be done in autumn or spring, before soil moisture become limiting, in restoration works.

B. rupestre seeds germinated to high percentages in a rather wide range of temperatures while *B. genuense* seeds in a rather narrow. Baskin & Baskin (1998) demonstrated that as seeds come out of primary dormancy, they germinate only over a narrow range of conditions, known as conditional dormancy. During the progression of dormancy loss, however, this range widens until seeds finally germinate over the full range of conditions possible for the population or taxon, at which point they are not dormant. Therefore, the accession of *B. rupestre* studied in this research were completely non-dormant at the moment when tests were set up, whereas *B. genuense* seeds were probably not. However, it is not possible to establish

whether the seeds studied were dormant or not when fresh. That is because it is not possible to exclude that dry storage, and therefore after-ripening, made the seeds come out of dormancy. Indeed, after-ripening is a common method used to release dormancy (Grime *et al.*, 1981; Hilton, 1984; Probert *et al.*, 1985b; Bewley, 1997; Probert, 2000; Leubner-Metzger, 2003; Kucera *et al.*, 2005; Bair *et al.*, 2006).

In any case, the purpose of the research was to test germination in stored seeds and, therefore, it is possible to state that air-dry seeds from central Apennines germinate to high percentages in a rather wide range of temperatures.

Some authors (Ratcliff, 1961; Newman, 1963) hypothesized that after-ripening is a mechanism preventing premature germination in dry habitats. The same explanation may be applied to the characteristic, although not very pronounced, response to dry storage evident in certain autumn-germinating perennial grasses such as *Festuca ovina*, *Koeleria cristata* and *Poa compressa* (Grime, 1981). The possibility must be considered, therefore, that in certain species a major effect of delayed ripening and germination is to facilitate seed burial.

In conclusion, it is important to emphasize the fact that seeds were after-ripened before being tested for germination. Therefore, the findings of this study describe the germination behavior of air-dry seeds of the grasses studied. Such behavior could be substantially different in fresh seeds.

LIGHT

Light was found to significantly enhance germination in *B. genuense* and in three populations of *B. rupestre* (Tab. 5).

Nondormant seeds of many species germinate equally well in light and darkness (Baskin & Baskin, 1988), those of others germinate to higher percentages in light than in darkness (Grime *et al.*, 1981; Probert 1985a; Baskin & Baskin, 1988), and those of a relatively few germinate to higher percentages in darkness than in light (Hammouda & Bakr, 1969; Hilton, 1982; Thanos *et al.*, 1992). In this study germination response to light was tested only at 20°C and always using the same kind and intensity of radiation. For this reason it is not possible to verify the effect of other factors which were found to modify the germination response to light, such as temperature (Thompson *et al.*, 1977; Bewley & Black, 1982; Probert *et al.*, 1985c), the spectrum of light applied (Kendrick, 1976; Ginzo, 1978; Bewley and Black, 1982; Hilton, 1982, 1984; Probert *et al.*, 1985a), the doses of photons (Thompson, 1989), and the photoperiod applied (Evenari, 1965).

Light is an extremely important factor in releasing seed from dormancy (Bewley and Black, 1994), although there is an underlying dark dormancy in many

species which disappears with time. Therefore, the fact that *B. genuense* seeds studied showed a so deep light requirement for germination could supports the hypothesis that seeds possibly had not completely lost dormancy at the time when germination tests were set up. In addition, light was found to have a major role in breaking seed dormancy in the majority of grass species (Simpson, 1990). For all these reasons, in order to establish whether a species requires light to germinate or not, seeds need to be tested in light and darkness when they are freshly matured and at regular intervals during the dormancy-breaking period, because their light requirement may change as they come out of dormancy (Baskin & Baskin, 1998). In any case, germination response to light and its effect on the release of dormancy are very variable. Grime (1981) found that in many species the difference applies to freshly matured seeds within the same seed collection, and it is known that seeds removed from the same inflorescence may exhibit marked differences in light requirement (Cavers & Harper 1966). Obviously, it would have been very interesting to test germination of freshly-collected seeds but, since the major purpose of this research was testing the suitability of autochthonous germplasm to multiplication and usage for environmental restoration, it was important to test seeds reproducing the conditions in which they will possibly be used in restoration projects. The theoretical findings of this study suggest that in the case of a large scale production of these seeds, sowing depth, which is related to the light requirements of seeds, could be controlled by the use of multiplication parcels to select the optimum burial depth for different seed populations of different species and consequently it could be reduced in seeds showing a light requirement for germination. This could maximize seed germination and therefore seed production.

OUTER COVERING STRUCTURES

The removal of outer covering structures did not increase final germination in *B. rupestre* seeds, it just increased the speed of germination (Tab. 6). Conversely, previous studies demonstrated that the presence of palea and lemma usually reduces the germination in seeds of grasses (Roberts, 1961; Hagon, 1976; Mott, 1974; Martin, 1975; Probert *et al.*, 1985d). Indeed, they mechanically restrict germination, reduce oxygen transport to the embryo (Delouche, 1956; Vose, 1956; Roberts, 1962; Stokes, 1965; Mott 1974), reduce imbibition or prevent the leaching of an inhibitor (Hagon, 1976). It has been found that the removal of the glumes and/or palea and lemma, as well as selective surgical treatments applied to otherwise intact seeds, reduced the level of dormancy in seeds of grasses and cereals (Roberts, 1961; Hagon, 1976; Mott, 1974; Martin, 1975; Probert *et al.*, 1985d). Probert *et al.* (1985d)

found that coat removal increased both rate and final percentage germination in *Dactylis glomerata*. Other studies demonstrated that palea and lemma reduce germination through different mechanisms, restricting the uptake of oxygen by the embryo (Mott, 1974), limiting gas exchange (Frank & Larson, 1970), acting as a mechanical barrier to the expanding embryo (Frank & Larson, 1970), releasing inhibitory substances (Hagon, 1976).

The evidence that *B. rupestre* seeds do not require the removal of outer covering structures has to be considered as an advantage for the purpose of this research. Indeed, this has positive consequences on restoration works. Indeed, if seeds of a species are found to not require this treatment, the extraction of seeds from palea and lemma will be not necessary and, thus, all the cleaning process will be quicker and, consequently, less expensive. Furthermore, removing the covering structures could also increase the risk of infection in seeds sown in the soil, especially if germination is delayed by low temperatures.

Conclusions

Germination was not problematic for studied seeds, since they were able to germinate to high percentages in a rather wide range of temperature. *B. rupestre* seeds did not require the removal of palea and lemma and it could allow to use a faster and less expensive extraction process.

As regards the light requirement for germination found in both species studied, it suggests that it will be important to not exceed in sowing depth.

So, it can be stated that seeds of the autochthonous populations studied can be easily multiplied and successfully used for marginal areas improvement, protecting the genetic purity of local populations. This aspect is very important for the conservation of habitats (Directive 92/43/EEC) and their restoration in Natura 2000 Network sites (Biondi *et al.*, 2012 and 2012a). Indeed, considering the *Brachypodium rupestre* and *B. genuense* ability to stop or reduce the evolution of grassland dynamic processes, they can be used in the recovery of particular habitats such as road embankments or hilly slops affected by erosion risks in order to maintain the stability in spatial and temporary terms and thus, reducing their management by men.

Moreover, the theoretical findings of this research will be really helpful to establish the best technical protocols for seed extraction, multiplication and seeding of studied species.

In this way, it could be possible to define germination protocols of herbaceous non food plants that could be used in the development of alternative agricultural activities and in the management of the environment. Such activities could represent new development per-

spective of mountain and hill areas. This would respond to the aims of important International conventions, such as The Convention on Biological Diversity (CBD), and to the Sustainable Development logic.

The reformed Common Agricultural Policy (CAP) has a "greener" and more equally subdivided first pillar and a second pillar more centred on competitiveness and innovation, climate change and environment. At national level, the National Strategic Plan for Rural Development 2007-2013 has been notified in 2009 and reformed on the basis of the European Plan for the economic relaunch which aims to: improve the competitiveness of agriculture and forest sectors; improve the environment and the countryside; improve the quality of life in rural areas and diversify rural economy. In particular, the II Axis, concerning the improvement of the environment and the countryside, considers: agri-environmental payments, Natura 2000 subsidies (to compensate costs and income losses due to the restrictions in the use of wood and forest imposed by 79/409/EEC and 92/43/EEC); non productive investments (investments that valorize protected areas in terms of public utility). Another important aspect concerns the support to agriculture through the Rural Development Plan (RDP) proposals for 2014-2020 in High Nature Value farmlands. In this context, semi-natural grasslands produced by anthropic activity play a very important role (Galdenzi *et al.*, 2011 and 2012). All these practices will be crucial through RDP payments not only for the respect of good agricultural practices but above all for the conservation and the recovery of biodiversity and of the most typical landscapes. The authors believe that financing the recovery of autochthonous germplasm must be considered in these plans and that the diffusion of non native species and varieties must be stopped. Indeed, they led to the genetic erosion of biodiversity. For this reason, a commerce of seeds of autochthonous non food herbaceous species it should be supported, at least within the EU.

Acknowledgements

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