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Research Paper

Effects of environmental factors on seed germination and seedling establishment in bilberry (*Vaccinium myrtillus* L.)

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ABSTRACT

The bilberry (*Vaccinium myrtillus* L.) is a spontaneous dwarf shrub with potential for national and international markets due to food and medicinal uses of its fruits, being considered an important source of sugars, antioxidants, vitamins and minerals. Request for bilberry fruits are on the rise, but the land area covered in bilberry bushes and locally collected berries have declined in Central Italian Apennine regions in recent last decades. The objective of this study was to assess the most suitable conditions for *in vivo* and *in vitro* seed germination in order to improve the reproductive performance of this species under artificial and controlled environments. Freshly matured bilberry seeds were tested for germination before and after cold stratification and air-room storage under different light conditions and temperatures. Seeds were then *in vivo* and *in vitro* germinated on different soil types and culture media. Fresh seeds showed a better germination capacity (76%) than stratified ones, being 16-h photoperiod and air temperatures of 22.5 °C the optimal conditions for germination. At 25 °C the germination rate decreased to 66% and very few seeds (8%) germinated at 15 °C. Darkness always had a negative influence on the germination capacity. However, after cold stratification germination increased significantly at 15 °C, both in light (51%) and darkness (24%); thus, seeds of this species are conditionally dormant at maturity. Although the effect was different depending on temperature and light condition, cold seed stratification had an overall negative effect on all the considered germination parameters, and seed viability was strongly reduced after storage at room temperature for 90 days. Soil type and culture media did not significantly affect the final germination percentage (≈62 and 78%) in the *in vivo* and *in vitro* trials performed on fresh non stratified seeds, respectively. Nevertheless *V. myrtillus* turf (*in vivo* experiment) and modified MS medium (*in vitro* experiment) gave rise to the most germination in the shortest time. Temperature proved to be the most influential physical factor on bilberry seed germination. Seedlings obtained from *in vivo* experiments showed good development.

1. Introduction

Bilberry (*Vaccinium myrtillus* L.) is a key species of wild Northern Europe mountain vegetation but it's only found on very acidic, humic soils throughout the temperate and subartic regions of the world (Nestby et al., 2010). In Italy the species finds its natural habitat from hilly areas to high altitudes above the tree-line in the Alps and in the north-central part of the Apennines. However, its optimal range is between 1500 and 2000 m where bilberry bushes grow both in open habitats and in the understory of conifer, chestnut or beech-dominated forests (Woodward, 1986; Dondini and Vergari, 2009).

This species has a great potential for the market because its fruits are an important source of sugars, vitamins, minerals and, above all, anthocyanins, which give these berries very high antioxidant and antiradical properties. Bilberries are used commercially for fresh consumption or processed (*i.e.* juices, jams, food supplements, among others) and medicinally (control of venous insufficiency and improving of night vision) (Beccaro et al., 2006; Ichiyanagi et al., 2006; Zafra-Stone et al., 2007; Lätti et al., 2008; Giovanelli and Buratti, 2009; Persson et al., 2009; Jovancević et al., 2011; Uleberg et al., 2012; Barizza et al., 2013).

Bilberries are extremely difficult to grow because of their reduced

Abbreviations: VMT, *Vaccinium myrtillus* turf; PBAS, commercial peat-based acid soil; D_{50}, time required for 50% of viable seeds to germinate; LAG, time at germination onset; G_{max}, final germination percentage; MGT, mean germination time

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environmental adaptability. They are rarely cultivated due to their low productivity, associated to the very small size of the non clustered fruits, compared to blueberry (*V. corymbosum* L.) which has larger berries born in corymbs (Ritchie, 1956). Fruits are mostly collected from wild plants growing on accessible lands throughout northern and southern Europe. In Italy, bilberries are mainly collected in natural environments of the Apennines, where they grow abundantly and picking of wild berries has been an important source of additional family income since the 1960s (Ronchieri and Mazzei, 1997). Recently, the picking of berries in Tuscany has been ruled according to the Regional Forest Law 39/2000 (ARSIA, 2005).

The average yearly bilberry production in Italy has been estimated to be about 6000–8000 tons, but there are no data about plant yield (g/plant), which can differ greatly depending on snow cover in winter and frost in spring. According to Nestby et al. (2010) productivity in northern Europe varies between 171 and 340 kg/ha. The estimated Italian area of production (about 5,000 ha) is mainly concentrated in the provinces of Pistoia, Lucca and Modena (ARSIA, 2005). Interestingly, data recently obtained by our research team highlighted a much higher anthocyanidin content in Tuscan bilberries than in other Italian specimens (Ancillotti et al., 2015). In Tuscany, the average price for fresh bilberries is about 6 Euro per kg (personal communication) with a total turnover of hundreds of thousands of Euros (Ronchieri and Mazzei, 1997). Requests for bilberry are on the rise due to ongoing interest in nutritional and nutraceutical properties of locally produced fruits, but the annual amount of collected wild berries has significantly declined in Tuscany in these last few decades. Possible reasons of the reduction in land area covered in bilberry bushes (bilberry-dominated vegetation) may be associated with present forest management (characterized by a reduced number of cuts compared to the past), a near disappearance of sheep grazing (which was selective in favor of *V. myrtillus* versus its vegetal competitors), and climatic changes (Ronchieri and Mazzei, 1997; Welch, 1997; Gerdol et al., 2013; Patsias and Bruelheide, 2013). Rochieri and Mazzei (1997) suggested that reduced snowfall and increased average yearly temperatures have favored the gradual shift of bilberry towards higher altitudes, causing its areal reduction. Furthermore, the so-called 'false bilberry' (*V. gaultherioides* Bigelow) is widely spreading as a competitor in *V. myrtillus* dominated areas. This advancing front of false bilberry hinders selective berry harvesting with combs and is cause of concern for the long term survival of bilberry.

V. myrtillus invades and regenerates both agamically from buds on the roots and gamically by seed (Ritchie, 1956). *Ex novo*-originated bilberry bushes develop only by seed dispersal and germination. The species generally produce many fruits and is thought to have high fecundity, moreover berries are attractive to animals that favor long-distance seed dispersal strategies (Eriksson and Fröborg, 1996). Nevertheless, unregulated human collection of berries represents a risk for natural seed dispersal away from the parent plant by both animals and environmental factors. 'Windows of opportunity' has been suggested as the most appropriate pattern of recruitment of bilberry seedlings among clonal plants (Eriksson and Fröborg, 1996; Albert et al., 2004), but clearly it depends on a supply of fresh seeds for its success. In Canada and in Northern Europe *V. myrtillus* is considered to reproduce largely vegetatively and seedlings are rare (Perttula, 1941; Ritchie, 1956; Flower-Ellis, 1971; Miller and Cummins, 1987; Hester et al., 1991; Vander Kloet and Hill, 1994; Welch et al., 1994; Welch et al., 2000; Ranwala, 2001; Miller and Cummins, 2003; Ranvala and Naylor, 2004).

Despite the success of vegetative reproduction, a lack of recruitment of new genetic individuals poses risks for this species. Under natural conditions, the processes of seed germination and seedling development of bilberry are hampered by several factors such as fluctuating humidity, temperature and light, and long germination processes take place and low rates of seedlings are obtained (Baskin et al., 2000). To satisfy the demand for seedling production of *V. myrtillus*, it is necessary to develop new procedures for the production of vegetative materials of this species. Few studies have reported propagation from sexual seeds of this genus (Baskin et al., 2000; Welch et al., 2000), while on the other hand, there are no known ***in vitro*** studies on seed germination. Currently, only some aspects have been studied about the morphology, storage and germination of the seeds (Vander Kloet, 1983; Ranwala and Naylor, 2004); light requirement and/or cold stratification (Ritchie, 1956; Mallik and Gimingham, 1985;); and seedling establishment on different sward types (Welch et al., 2000).

The present study regards the assessment of the most suitable conditions for *in vivo* and *in vitro* seed germination in order to improve the reproductive performance of this species under artificial and controlled environments. Particularly, the effect of air temperature, light conditions, cold stratification/storage and substrate on seed germination parameters were investigated. Acclimation of *in vivo* obtained seedlings was also analysed.

2. Materials and methods

2.1. Seed source

Seeds for the different experiments were obtained from ripe berries collected by hand in the Abetone area (Central Apennine – Tuscany) during July-August. Berries were carried to the pomology laboratory at the Department of Agri-Food and Environmental Science (Florence University), and crushed gently to release the seeds, which were accurately washed free of fruit material and dried in open air at room temperature for four days prior to initiation of studies.

2.2. Experiments

A first preliminary experiment (Experiment I: seed germination under different environmental conditions) was performed in order to assess the most suitable temperature and light conditions for the germination of both freshly matured seeds, as well as stratified and stored seeds. A subsequent experiment (Experiment II: *in vivo* and *in vitro* seed germination on different soil types and culture media, respectively) regarded the germination of bilberry seeds tested *in vivo* on different soil types and *in vitro* on various culture media and, finally some parameters related to seedling acclimation and hardening were analysed (Experiment III: seedling transplant and hardening off).

2.2.1. Experiment I: seed germination under different environmental conditions

Seeds (n = 14,400) of bilberry were randomly divided in lots of 400 seeds (split into 4 replications of 100 seeds each), and used to test the effect of air temperature (15, 22.5 and 25 °C) and light/dark conditions (16 h light/8 h dark and 24 h dark) on i) non stratified fresh seeds (control), ii) stratified seeds at 4 °C for 15, 30, 60 and 90 days and iii) stored seeds at air-room temperature (20 °C) for 90 days. Falcon flasks were used for seed storage and cold stratification.

Seeds were cultured in 90-mm-diameter Petri dishes on a sheet of filter paper moistened with deionised water. Petri dishes were incubated in growth cabinet with a precision thermoregulator (± 0.5 °C) at temperature regimes of 15, 22.5 and 25 °C under fluorescent light (35 μmol m^{-2} s^{-1}, provided by 75 W Silvana lamps) with 16-h photoperiod and in continuous darkness. Darkness was obtained by placing dishes in closed cardboard boxes.

Germination was assessed every alternate day and continued until there were no newly germinated seeds for three consecutive days. The criterion for germination was emergence of the radical to greater than 2 mm.

2.2.2. Experiment II: in vivo and in vitro seed germination on different substrates and culture media

Air-dried, freshly matured seeds (n = 4800) were used for these

Table 1
Physicochemical properties of soil samples collected in *V. myrtillus* growing area (Abetone mountain) and commercial peat.

Soil properties	*V. myrtillus* turf (VMT)	Commercial peat-based acid soil (PBAS)
Organic carbon %	5.3	30.0
Humic and fulvic carbon %	n.d.	7.0
Organic nitrogen %	0.4	0.6
Carbon-to-nitrogen ratio	13.4	50.0
Total copper mg kg^{-1}	n.d.	150.0
Total zinc mg kg^{-1}	n.d.	500.0
pH in water	5.4	6.0
pH in KCl	3.7	n.d.
Peat to total %	n.d.	50.0
Salinity dS m^{-1}	n.d.	0.6
Texture:		
Sand %	56.6	n.d.
Fine sand %	24.2	n.d.
Medium sand %	14.8	n.d.
Large sand %	61,0	n.d.
Silt %	27.6	n.d.
Clay %	15.8	n.d.

tests. Seed surface disinfection was carried out in a laminar flow chamber as follows: rinsed with ethanol 70% (v/v) for 1 min, dipped in a mixed solution of sodium hypochlorite (0.525%) and Tween 20 solution (0.1%) for 15 min and, finally, rinsed three consecutive times with sterile distilled water.

Natural, non sterilized *Vaccinium myrtillus* soil (*i.e.* soil collected under spontaneous *V. myrtillus* bushes, hereafter indicated as *V. myrtillus* turf – VMT) and four different autoclaved substrates were used as culture media for the *in vivo* germination experiment: commercial peat-based acid substrate (PBAS), commercial peat-based acid substrate and perlite 1:1, sand of vulcanic origin, and commercial peat-based acid substrate mixed with sand 1:1. Soil mixtures were prepared on a volume basis. Physico-chemical properties of VMT and PBAS are reported in Table 1. Substrates were kept constantly moist with non calcareous mountain spring water (pH at the source 6.3; specific electrical conductance 15.2 µS/cm; fixed residue at 180 °C 14.0 mg/l; free carbon dioxide 4.0 mg/l; hardness 0.55 °F; sodium 1.0 mg/l) collected from the same area of spontaneous plant growth.

The culture media used for *in vitro* germination were MS (Murashige and Skoog, 1962) added with 0 and 24.6 µM 2iP and modified MS medium (Economou and Read, 1984) containing different doses of 2iP (0, 24.6 and 49.2 µM) or Zeatin (0, 9.1, 18.2 µM), supplemented with 10 g/l of sucrose. The medium was adjusted to pH 4.8, solidified with 6.5 g/l agar (Difco-Bacto) before autoclaving for 20 min at 121 °C and 108 kPa. Forty seeds were placed in 170-ml glass jars for each of the ten replications.

Taking into account both the cumulative germination percentage and the time of germination onset determined in the preliminary experiment, the best assessed growing conditions (*i.e.* 16 h photoperiod of 35 µmol m^{-2} s^{-1} photon flux at 22.5 ± 0.5 °C air temperature) were adopted for this experiment in a phytotron (relative humidity of 80%) and in a growth chamber for *in vivo* and *in vitro* germination trial, respectively. Rising of the cotyledons above the ground was the criterion for germination assessment.

2.2.3. Experiment III: seedling transplant and hardening off

A sub-set of 200 seedlings of 1.0–1.5 cm of height selected randomly among those obtained from *in vivo* experiments (Experiment II) was transplanted from the glass jars to polystyrene trays with individual cells (2.5 cm ø) containing commercial peat-based acid substrate. Trays were kept in the phytotron for 12 weeks at a temperature range of 22.5 ± 0.5 °C with a photoperiod of 16 h light (300 µmol m^{-2} s^{-1}) and 8 h dark and watered at two-day intervals with mountain spring water. In this study the percentage of viable seedlings, number of leaves and seedling length were assessed.

The seedlings that attained a length of 3–4 cm, with 8–10 short internodes and 10–14 leaves were transferred to a greenhouse at 22 °C and 80% air humidity. After one week, the seedlings were replanted in plastic pots (10 cm ø) with a substrate of PBAS, perlite and sand 2:1:1 (on volume basis) and irrigated weekly. After 12 weeks the percentage of viable seedlings and the number and length increase of axillary shoots were quantified.

2.3. Statistical analysis

The cumulative germination count of each bilberry seed lot was fitted to a four-parameter Hill function (4-PHF; Eq. (1)) previously reported by El-Kassaby et al. (2008) as a mathematical representation and parameters extraction of seed germination:

$$y = y_0 + \frac{ax^b}{c^b + x^b} \tag{1}$$

In Eq. (1), y is considered to be the percentage of cumulative germination at time x, y_0 is the intercept on the y axis (≤ 0), a is the asymptote, or maximum cumulative germination percentage, which is equivalent to germination capacity, b is a mathematical parameter controlling the shape and steepness of the germination curve and c is the “half-maximal activation level” measured in days and represents the time required for 50% of viable seeds to germinate (D_{50}). This latter parameter was considered only for maximum cumulative germination percentages greater than 30%. Following extraction of a, b, c and Y_0, from 4-PHF, the time of germination onset (LAG) and the final germination percentage (G_{max}) were also computed using the following equations:

$$LAG = b\sqrt{\frac{-y_0 c^b}{a + y_0}} \tag{2}$$

$$G_{max} = y_0 + a \tag{3}$$

The mean germination time (MGT) was calculated for each lot using the formula cited by Ellis and Roberts (1980):

$$MGT = \Sigma(n \times D)/\Sigma n$$

where n = number of seeds newly germinated at time D;
D = days from the beginning of the germination test,
Σn = final germination.

The whole research work was designed as a completely randomized design. Data were processed by ANOVA analysis of variance and means were compared using Duncan’s multiple range test with a confidence level of 99%. Percent values were arcsine transformed prior to analysis.

3. Results

3.1. Experiment I: seed germination under different environmental conditions

The significance of single and combined effects of factors for the studied parameters obtained in Experiment I is shown in Table 2. Most germination parameters of bilberry were significantly affected ($p < 0.01$) by temperature, photoperiod, stratification and conservation time; only mean germination time and time taken to 50% germination were not influenced by temperature and/or photoperiod (Table 2). Furthermore, several significant effects of combined factors were observed for many parameters.

A significant temperature x photoperiod interaction occurred only for the time of germination onset. The lowest values (16.8 and

Table 2
Significance of singles and combined effects of factors for the studied parameters in Experiment I.

Effect	G_{max}	MGT	LAG	D_{50}
Temperature (T)	**	ns	**	ns
Photoperiod (P)	**	**	**	ns
Seed treatment (S)	**	**	**	**
T x P	ns	ns	*	ns
T x S	**	**	*	ns
P x S	**	*	*	ns
T x P x S	*	*	ns	**

ns = non-significant.
* significant for $p < 0.05$.
** significant for $p < 0.01$.

Table 3
Effect of interactions between temperature (T), photoperiod (P) and seed treatment (S) on the studied parameters in Experiment I.

Factor T	P	S	G_{max} (%)	MGT (days)	LAG (days)	D_{50} (days)
15 °C	16 h L/8 h D	Control	10.0 d	20.6 a	20.0	–
15 °C	16 h L/8 h D	Treated	51.1 b	26.7 bc	21.5	4.3 ab
15 °C	24 h D	Control	6.7 d	23.5 ab	23.5	–
15 °C	24 h D	Treated	23.5 c	27.5 bcd	22.4	2.7 a
22.5 °C	16 h L/8 h D	Control	81.7 a	19.7 a	14.0	4.2 ab
22.5 °C	16 h L/8 h D	Treated	49.8 b	23.3 ab	18.9	3.4 ab
22.5 °C	24 h D	Control	73.3 a	19.6 a	12.5	6.2 b
22.5 °C	24 h D	Treated	23.1 c	30.8 d	24.9	3.0 a
25 °C	16 h L/8 h D	Control	83.3 a	27.9 cd	14.5	9.5 c
25 °C	16 h L/8 h D	Treated	48.0 b	23.5 ab	17.3	4.3 ab
25 °C	24 h D	Control	48.4 b	25.4 bc	17.0	4.7 ab
25 °C	24 h D	Treated	20.1 dc	25.6 bc	21.6	2.7 a
Significance			*	*	ns	**

Averages represent the mean values for untreated seeds (control) and treated seeds (cold stratification for 15, 30, 60, 90 days and air room stratification for 90 days). Mean separation within columns by Duncan's multiple range test ($p < 0.05$ and $p < 0.01$). Percentages were arcsin-transformed before analysis. ns = non-significant.
* significant for $p < 0.05$.
** significant for $p < 0.01$.

18.1 days) were observed when seeds were germinated under light condition at 25 and 22.5 °C, respectively; conversely, a detrimental effect was observed when darkness and temperature of 15 and 22.5 °C were combined together (22.6 and 22.9 days, respectively).

Temperature x seed treatment interactions were significant for all parameters except for D_{50} (Fig. 1). Germination was highest for fresh seeds at the two highest temperatures (77.5% and 65.9% for 22.5 and 25 °C, respectively); at these regimes seed stratification and conservation had a reductive effect, while at 15 °C such treatments increased G_{max} up to more than 4 times (37.3%) compared to the control (8.3.3%). Lower MGT and LAG (19.7 and 13.2 days, respectively) were obtained when fresh seeds were germinated at 22.5 °C, while the highest values were observed with the combination of either 15 and 22.5 °C with treated seeds (27.1 and 21.9 days for MGT and LAG, respectively).

Photoperiod by seed treatment interaction showed that a significant lower percentage of treated seeds (20.0%) germinated when incubated under continuous darkness conditions. The absence of light had a strong negative effect on MGT and LAG of treated seeds, as well (28.0 and 23.0 days, respectively). Germination parameters were best in presence of light for fresh seeds (58.3%, 22.7 days and 16.2 days, for G_{max}, MGT and LAG, respectively).

Finally, T x P x S interaction evidenced that the best combination of environmental conditions for bilberry seed germination, *i.e.* the highest G_{max} and lowest MGT, was the following: temperature of 22.5 °C, presence of light and fresh non stratified seeds (Table 3). Under light condition, fresh seeds showed a very high germination rate also at 25 °C, although MGT increased significantly (27.9 days). Compared to

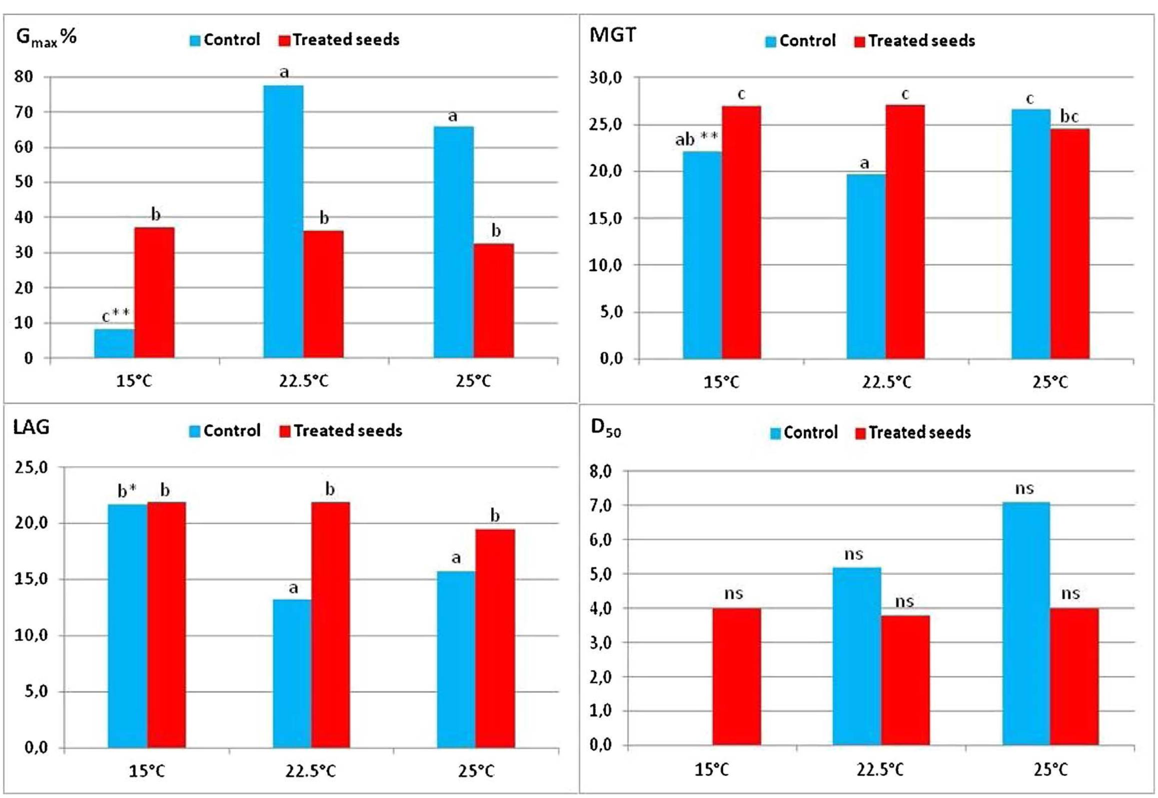

Fig. 1. Effect of T x S interaction on the studied parameters in Experiment I. Averages represent the mean values for untreated seeds (control) and treated seeds (cold stratification for 15, 30, 60, 90 days and air room stratification for 90 days). Mean separation within boxes by Duncan's multiple range test ($p < 0.05$ and $p < 0.01$). Percentages were arcsin-transformed before analysis. ns = non-significant; * significant for $p < 0.05$; ** significant for $p < 0.01$.

the control, seed treatment showed to exert a positive effect on seed germination only under a temperature regime of 15 °C, both in presence or absence of light (51.1% vs. 10.0% and 23.5% vs. 6.7% with a 16-h photoperiod and in complete darkness, respectively). Otherwise, G_{max} of treated seeds was less than, or in a few cases similar to, those obtained for fresh non stratified seeds.

3.1.1. Temperature and light requirement for germination of fresh non stratified seeds

Overall germination percentage was highest at 22.5 °C (77.5%) and lowest at 15 °C (8.3%). Light condition had a strong influence on seed germination, with a photoperiod of 16 h light giving the highest G_{max} values (58.3% against 42.8%). The germination capacity at 15 °C was always very low, independently of light conditions, thus distorting the corresponding MGT, which was 22.1 days on average. Germination of seeds incubated in the presence of 16 h of light ranged from 81.7% to 83.3% at 22.5 and 25 °C, respectively, but MGT sharply increased from 19.7 to 27.9 days with increasing temperature. The germination rate was strongly reduced in darkness, and the effect was maximum at 25 °C.

At incubation temperatures of 22.5 and 25 °C, germination of fresh seeds of *V. myrtillus* commenced 16–22 days after setting to germinate and finished between days 29–36, depending on germination conditions, while a delay of onset by 2–4 days and the end of the germination process within 2–9 days from onset was observed at 15 °C. The temperature regime strongly influenced the lag phase, which is an important parameter to determine quality of seed lots. The lag period was reduced from 21.7 to 13.2 days by raising the temperature of incubation to 22.5 °C, but it increased again up to 15.7 days at 25 °C. On the contrary, light conditions were without effect on the lag phase.

Time taken to 50% germination is also considered as a remarkable parameter in seed germination tests. According to our results this period was reduced to 5.2 days at 22.5 °C ($p < 0.01$), while the photoperiod was not effective on this parameter, regardless of temperature regime.

3.1.2. Effect of different stratification and storage treatments

ANOVA analysis showed that "seed treatment" (fresh vs differently stratified and conserved seeds), as a principal factor, exerted a very significant effect on all the studied parameters (Table 2). In this section, we report the results related to the effect of different stratification times and conservation within the set of treated seeds, taking into account also their interactions.

Both single and combined effects of factors were highly significant for the studied parameters, with the exception of D_{50}, as from ANOVA analysis performed only on the sub-set of treated seeds. The most outstanding results are here reported taking into account the T x P x S interaction.

G_{max} values showed a different trend depending upon the photoperiod: in presence of light the peak values (over 60%) were observed when seeds were stratified for 15–30 days, then values slowly decreased with time of conservation, while in darkness G_{max} never exceeded 40% and it decreased sharply at 25 °C when storage period was longer and when seeds were conserved at air room temperature. Generally, G_{max} was minimum and MGT values were maximum (over 30 days) for seeds stored at room temperature for 90 days. Conversely, MGT and LAG of differently stratified seeds showed an oscillating trend varying between 21.6 and 29.7 days, and 16–26.3 days, respectively. Independently of the stratification duration, lower MGT and LAG were observed at 22.5 and 25 °C in presence of 16-h photoperiod (data not shown).

3.2. Experiment II: in vivo and in vitro seed germination on different soil types and culture media, respectively

Cumulative germination of seeds cultured *in vivo* in different soil types showed differences that were not statistically significant, ranging between 55.0 and 75% (Table 4). On the contrary, MGT and lag phase differed significantly, and were shortest in VMT and longer in presence of commercial PBAS or PBAS combinations with perlite or sand (20.0 versus 30.6, 28.6 and 27.3 days, respectively for MGT; 15.0 versus 26.0, and 20.5 days, respectively for LAG). Seedlings propagated in sand and a mixture of PBAS and sand grew taller (average height of 1.3 cm in 7 days) with smaller leaves; conversely, seedlings developed in PBAS and PBAS plus perlite were shorter (average height increase of 0.8 cm in 7 days) and with large leaves.

Table 4
Effect of soil type on *in vivo* germination of fresh seeds as from Experiment II.

Soil type	G_{max} (%)	MGT (days)	LAG (days)	D_{50} (days)
V. myrtillus turf (VMT)	75.0	20.0 a	15.0 a	4.2
PBAS	55.0	30.6 c	26.0 c	3.5
PBAS and perlite 1:1	56.6	28.6 c	21.2 b	4.7
Sand of vulcanic origin	60.0	23.9 b	19.2 b	2.7
PBAS and sand 1:1	60.0	27.3 c	20.5 b	4.0
Significance	ns	**	**	ns

ANOVA test: ns = non-significant.
** = significant ($p < 0.01$). Mean separation within columns by Duncan's multiple range test ($p < 0.01$). Percentages were arcsin-transformed before analysis.

When seeds were cultured *in vitro* the range of germination percentage oscillated between 66.7 and 88.9%, values which were approximately 15–20% higher than those observed with *in vivo* cultured seeds (Table 5). Concentration of salts and phytohormones did only affect MGT and lag phase, which were shortest when seeds were obtained on MS medium with or without 2iP. On the other hand, culture media influenced the seedling quality. Generally, seedlings grown on modified MS medium, with or without the addition of phytohormones, had central leaves which became red colored with time. In absence of growth regulators or in presence of 2iP, independently of the type of basal medium, seedling were characterized by a greater height.

3.3. Experiment III: seedling transplant and hardening off

After 12 weeks in the incubation room, the viability percentage of seedlings transplanted to commercial peat-based acid soil was 91%. Seedling height ranged between 2.2 and 5.0 cm with an average of 2.8 cm, while the number of leaves varied from a minimum of nine to a maximum of 17 with an average value of 10.6. After 12 weeks in the greenhouse, 54% of the seedlings showed a vigorous growth with a mean production of 1.8 ± 0.7 of shoots and an increase in mean length of 4.7 ± 1.3 cm (Fig. 2), showing that substrates with abundant organic matter have a positive effect on the development of bilberry seedlings. Most of the survived seedlings (38%) were obtained from seeds germinated on VMT, while the remaining part (62%) were coming up from seeds on pure sand, PBAS or PBAS-based substrate mixtures. In other words, 92.5% of seedlings obtained on VMT survived over a period of 3 months in the greenhouse, while survival rates varied between 22.5 and 50% when seedlings developed on pure PBAS,

Table 5
Effect of culture media on *in vitro* germination of fresh seeds as from Experiment II.

Culture media	G_{max} (%)	MTD (days)	LAG (days)	D_{50} (days)
MS	78.3	22.7 a	16.3 a	3.8
MS + 24.6 μM 2iP	81.7	21.3 a	16.3 a	3.6
mod MS	68.6	30.3 c	20.6 bc	4.6
mod MS + 24.6 μM 2iP	77.5	29.6 c	22.2 c	4.0
mod MS + 49.2 μM 2iP	66.7	24.8 ab	19.7 bc	3.0
mod MS + 9.1 μM Zeatin	88.9	25.3 ab	19.0 b	3.0
mod MS + 18.2 μM Zeatin	80.0	27.5 bc	19.6 bc	4.5
Significance	ns	**	**	ns

ANOVA test: ns = non-significant.
** = significant ($p < 0.01$). Mean separation within columns by Duncan's multiple range test ($p < 0.01$). Percentages were arcsin-transformed before analysis.

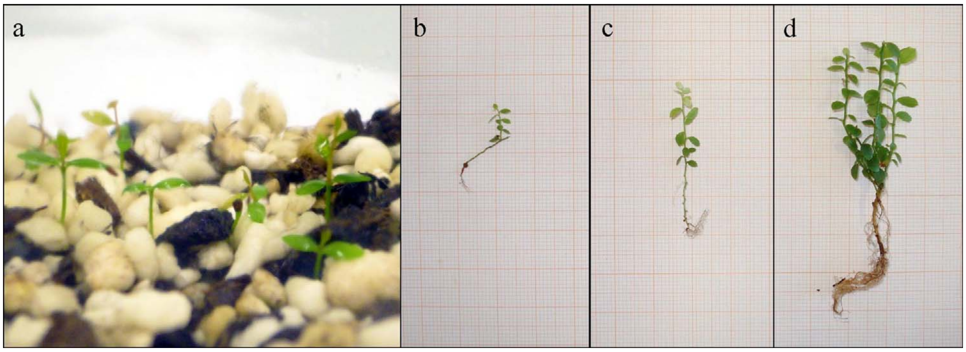

Fig. 2. Seedling development: a = seedlings grown in glass jar on PBAS and perlite 1:1; b = seedling at age of transplanting in polystyrene tray; c = seedling grown in polystyrene tray on PBAS; seedling grown in plastic pots on PBAS, perlite and sand 2:1:1.

mixture of PBAS with perlite, pure sand and PBAS mixed with sand.

4. Discussion

4.1. Experiment I: seed germination under different environmental conditions

Overall final germination of preliminary Experiment I was 39%, however the germination rate significantly differed depending on temperature regimes, light conditions and seed storage periods.

After five weeks of incubation in presence of light, 81.7 and 83.3% of the freshly matured seeds (control) of bilberry germinated at 22.5 and 25 °C, respectively, but at an incubation temperature of 15 °C only 10% of non stratified seeds germinated (Table 3). Under these latter conditions, an early process of necrosis took place in the roots associated with the stop of epicotyledonary growth.

These results agree with those obtained by Baskin et al. (2000) and Ranwala and Naylor (2004), who demonstrated that germination rate of *V. myrtillus* is greatest when seeds are incubated between 20 and 25 °C and in presence of light. Baskin et al. (2000) simulated mean daily maximum and minimum monthly temperatures near the soil surface during the growth season in Sweden and found germination rates of 91 and 84% at daily alternating temperature regimes of 20:10 and 25:15 °C, respectively and in presence of light, while at alternating temperature regimes of 15:5 °C germination percentage was reduced to 12%. According to Ranwala and Naylor (2004), bilberry seed germination occurred over the threshold of 15 °C, increased from 15 to 23 °C and sharply decreased thereafter; final germination was greatest (86%) at 21 °C with a photoperiod of 16 h of light. Mallik and Gimingham (1985) obtained a germination rate of 64% in presumably freshly matured seeds at 20 °C, but they did not report whether seeds were incubated in light or darkness. Jacquemart (1997) reported 42% germination of freshly matured seeds with 16 h day-length, but indicated vaguely a range of 15–25 °C diurnal temperature. Finally, Ritchie (1956) recorded 46% germination of apparently freshly matured seeds of bilberry in light at room temperature.

In our test, light conditions always positively affected G_{max}, in accordance with the results reported by Baskin et al. (2000) and Castro et al. (2012). Germination rate was significantly reduced in darkness, but deflation varied consistently with the regime of temperature, with minimum at 22.5 °C and maximum at 25 °C. In most cases, seedlings were characterized by chlorotic radicals and epycotyls and were apt to spin. Baskin et al. (2000), on the contrary, reported that none of the non stratified seeds of bilberry germinated in darkness, regardless of the temperature. Non stratified seeds of *V. myrtillus* from former Yugoslavia required a nine-day pretreatment of continuous exposure to red light before 90% of the seeds germinated in darkness at 25 °C (Giba et al., 1995). According to Correa (2002), seeds of some *Vaccinium* species could be classified as not affected by light because they germinate in light and dark conditions. However, the quantified differences between G_{max} in our experiment indicate that the light factor exerts a significant influence on the germination process. In turn, Finch and Leubner (2006) showed that in most seeds, germination is faster in conditions of light due to the presence of phytochrome in the seed embryonary axis.

Both the mean germination time and lag phase were shortest at 22.5 °C, but longer compared to the data reported by Ranwala and Naylor (2004).

Our results are only partially in agreement with those reported in previous studies of the same species. Unfortunately, the different methodologies applied for seed germination strongly limit the comparability with previous studies, which in some cases lack any information about light condition during incubation and state of fruit maturity during seed collection. Hartmann et al. (1997) and Bridgen (2001) highlighted the importance of finding the optimum state for seed collection because of the inaccurate development of immature embryos. Moreover, also seed size may account for some of the differences in reported germination of bilberry seeds, as many reports seem only to have considered large seeds (Ritchie, 1956; Jaquemart, 1997; Welch et al., 2000).

Both seed stratification and storage treatments had an overall negative effect on the considered germination parameters, however this effect was different depending on temperature and light conditions of seed incubation and was particularly evident with regard to G_{max}. Germination rate of treated seeds decreased by almost 50% compared to the control at 22.5 and 25 °C, while it nearly quadrupled at 15 °C (36.4% vs 77.5%, 32.6% vs 65.9% and 37.3% vs 8.3%, respectively). Percentage of germinated seeds reached its maximum value of 66.7% at 15 °C in presence of 16-h photoperiod when seeds were stratified for 30 days.

Although high germination percentages were obtained in light at 22.5 and 25 °C in the present study, these results do not necessarily mean that the seeds were non dormant. Actually, seeds could have been conditionally dormant and, as defined by Vegis (1964), they could germinate over a narrow range of conditions, which can be increased with appropriate treatments. A dormancy breaking treatment such as stratification for 30 − 60 days increased the range of temperatures over which germination occurred, causing seeds of bilberry to gain the ability to germinate at rather high percentages when incubated at 15 °C and in the dark. Thus, data obtained in the control treatments do not represent the maximum possible germination percentage and therefore seeds are conditionally dormant at maturity.

Baskin et al. (2000) considered *V. myrtillus* seeds as conditionally dormant at maturity as well. These authors reported a different behavior of seeds depending upon light-dark conditions during stratification. Germination of seeds stratified in light and incubated in light at 20:10 or at 25:15 °C was not significantly different from germination of non stratified seeds, but when seeds were stratified in darkness they germinated to significantly lower percentages than did non stratified seeds in accordance with our results. Similarly, stratification (both in light and darkness) increased the germination percentage of seeds at 15:5 °C, but only when they were placed in light; contrary to our data, stratified seeds in their study did not germinated in darkness. Thus, Baskin et al. (2000) suggested that germination at temperature regimes of 20–25 °C of stratified seeds declines not owing to stratification per se, but because of being in darkness during stratification. Discordant results have been reported also by Ritchie (1956) and Mallik and Gimingham (1985), according to whom germination percentage of seeds incubated at 20 °C, after three and five weeks of stratification at 0 °C, increased up to 64% and was only 6%, respectively, but light-dark conditions during stratification were not indicated. The implication given by Baskin et al. (2000) was that the first bilberry seeds to germinate in spring-early summer would be those on the soil surface, whereas seeds that overwintered and were covered before they could germinate have the potential of germinating in darkness; however, this would not happen until maximum day-time temperatures increased to 20–25 °C.

The negative effect of treatments on MGT and LAG was worst at 22.5 °C, nevertheless no data about these parameters have been reported by other authors in similar studies.

Collecting and storing dry *V. myrtillus* seeds could represent a possible management option to increase the number of seedlings of bilberry in case of unsuitable microsites for germination. However, in our study seed viability was strongly reduced after dry storage at room temperature for 90 days, contrary to previous reports by other authors. Germination of *V. myrtillus* seeds stored in cold moist conditions decreased linearly with time and was zero after 300 days (Ranwala and Naylor, 2004), but germination of seeds stored dry for one year was not appreciably different from that of fresh seeds (Ranwala and Naylor, 2004; Welch et al., 2000). A seed viability decrease could be related, according to Correa (2002), to seminal deterioration during storage, which produces strong changes in enzymatic amount and activity, degrading the nutritional reserves and, according to Pérez and Martínez (1994), because of alterations in the substances responsible of metabolism. Hence, the constant metabolic activity inside the seeds in the conditions of this study may have negatively influenced the process of seed germination of bilberries stored for 90 days.

Obviously, temperature proved to be the most influential physical factor on bilberry seed germination. Its effect is due to an ability to influence the activity of enzymes that regulate the rate of biochemical reactions occurring inside the seed after its rehydration. In bilberry seeds, 22.5 °C was the most suitable temperature at which the enzyme activity progressed efficiently, stimulating the highest percentage of germination in the shortest time.

4.2. Experiment II: in vivo and in vitro seed germination on different soil types and culture media, respectively

On average, almost 62% germination was observed on the five different types of soil. Although soil composition did not significantly affect G_{max}, *V. myrtillus* turf gave rise to the most germination in the shortest time, while D_{50} was the lowest on pure sand. Also in the *in vitro* experiment, culture media did not affect G_{max}. Germination reached almost 78% on average, but MGT, LAG and D_{50} values were similar to those obtained in the *in vivo* experiment. These results highlighted temperature and light as the most relevant variables to increase bilberry germination rate. Soil type and culture media, on the contrary, would mainly play a key role in the onset and fastening of the germination process. Our data showed that *V. myrtillus* turf texture soil was best for maximum and faster germination of seeds most likely as a result of soil acidity, good porosity and sufficient soil moisture. Obviously, these characteristics are part of the natural soil where bilberry grows. The beneficial effect of the sandy soil on seedling emergence was probably due to the optimization of several soil physical properties. Benvenuti and Macchia (1995) found that the time required for seed germination increased as a function of the physiological characteristics of the soil typologies, being the linear regression between D_{50} and soil air permeability highly significant. In soil, on the other hand, the sudden increase in oxygen consumption by germinating seed together with the limited potential for gaseous exchange creates a hypoxic environment in the immediate surroundings of the seed (Gulsham and Dasti, 2012). Thus, the decrease in emergence rate has been linked to poor gas exchange in the environment surrounding seeds more than to the actual quantity of oxygen present in the various soils (Benvenuti and Macchia, 1995).

4.3. Experiment III: seedling transplant and hardening off

Seedlings from *in vivo* germinated seeds showed good development when transplanted to a substrate containing PBAS, perlite and sand 2:1:1. During seedling growth, it was observed that the composition of the substrate was an important factor for the development of the cauline and roots. Clearly the substrate with abundant organic matter mixed with sand and perlite was very suitable for the hardening of bilberry seedlings. This result can be associated with the substrate features such as high porosity, high water retention, good aeration, low pH, fine texture along with mycorrhizae, largely responsible for seedling vigor (Valencia and Ramírez, 1993). Furthermore, the abundance of organic matter facilitates the development of a fibrous, thin, shallow root system, characterized by a shortage of root hairs (Malik and Cawthon, 1998), which helps the roots anchor and expand among the fine particles. Nevertheless, over a period of six months, just over 50% of seedlings survived in the greenhouse, 38% of which were deriving from seeds germinated in *V. myrtillus* turf.

Vander Kloet (1983) in a survey of seed and seedling morphology in the same species, quantified 40% germination in seeds placed on a 1:1 peat sand mixture in a misting chamber in the greenhouse at 22.5 °C, but no information was given about seedling development and survival. In previous studies carried out by Welch et al. (2000) seedling establishment was found to be greatly affected by sward type; many fewer germinations were observed on three types of peat (23%, 1% and 9%, respectively on moss grass, *Vaccinium* and *Nardus* peats) than on bare peat (73%). In the glasshouse (10–25 °C), germination rates were lower than in the growth chamber (20 °C), probably caused by the slower onset of germination in a cooler environment no or very few germinations were recorded in outdoor tests and these seedlings soon died. Thus, it is probably realistic to assume that successful seedling establishment represents the crucial step for expansion of bilberry populations.

Recruitment of new seedlings into the vegetation is essential for maintaining species rich plant communities. Hence it is of great importance to understand factors determining seedling recruitment. A number of studies have reported beneficial effects of mycorrhizal presence on seedling establishment and plant growth of different *Vaccinium* spp. (Scagel et al., 2005; Kosola et al., 2007; Vega et al., 2009; Arriagada et al., 2012). In natural conditions, mostly common under acid and infertile land conditions, wild *Vaccinium* plants spontaneously form mutualist symbiotic association with certain soil fungi, called 'ericoid mycorrhizae' (Scagel et al., 2005; Smith and Read, 2008; van der Heijden et al., 2015). Ericoid mycorrhizae would be specific to these species, due to their co-evolution with the native host plants growing in limiting edaphic conditions, allowing the plant to complete its life cycle (Brundrett, 2004). Moreover, as soil is a spatially variable substrate, through the ages, mycorrhizal fungi may have evolved biotypes adapted to specific soil conditions of a land area (van der Heijden et al., 2015). According to Scagel et al. (2005), in nursery production, *Vaccinium* plants can become naturally colonized by ericoid mycorrhizal fungi, however, colonization is sporadic and can be quite low depending on the plant genotype and production method. The benefits from root colonization by mycorrhizal fungi are thought to be highest when colonization occurs as early as possible during plant growth (Chang, 1994; van der Heijden, 2004), this means that inoculums should be present during radical emergence in seed germination. It can be hypothesized that in our experiment the established seedlings may have benefited from the mycorrhizal presence in *V. myrtillus* turf during seed germination that promoted seedling growth and vigor.

Seedling colonization requires at least two necessary conditions: seed availability and high rate of germination. Bilberry fruits collected in Central Italy for our experiments, resulted rich in seeds that showed a high capacity of germination in artificial environmental conditions (namely air temperature and photoperiod) similar to those observed in the natural conditions of the Tuscan Apennine at fruit ripening time. Such climatic conditions allow the immediate germination of seeds and their early and fast growth until Autumn. On the other hand, in different studies on mountain seed banks of various regions, authors found that bilberry seeds and seedlings were absent or scarce despite bilberry plants being prominent among the surface vegetation (Miles, 1973; Mallik et al., 1984; Hester et al., 1991; McFerran et al., 1994; Vander Kloet and Hill, 1994; Welch et al., 1994; Welch et al., 2000; Ranwala, 2001; Naylor, 2002; Miller and Cummins, 1985, 2003; Ranvala and Naylor, 2004). Possible reasons for the scarcity of bilberry seedlings are a low fruit set and low production of viable seeds, their dormancy status, their germination requirements and their longevity and survival in the soil (Miller and Cummins, 2003; Welch et al., 2004). Welch et al. (2000) reported that the main factors causing a rarity in *V. myrtillus* establishment seem to be the poor defenses and a very slow growth and weak competitive ability of the seedlings. Pakeman and Hay (1996) highlighted the failure to break seed dormancy as responsible for low density of bilberry seedlings. Hill and Stevens (1981) hypothesized a rapid decline in viability of *V. myrtillus* seeds in the soil. This rapid decline could result from degradation of the seed coat, which Granström (1987) observed and ascribed to fungi, and also from germination occurring soon after dispersal in conditions inimical for seedling growth. Although some differences have been found in the number of viable seeds (Granström, 1987; Vander Kloet and Hill, 1994; Cippollini and Stiles, 1992), production of germinable seeds would appear not to limit the number of bilberry seedlings in natural conditions (Welch et al., 2000; Ranwala and Naylor, 2004). The apparent scarcity of new seedlings of *V. myrtillus* despite the investment of biomass into fruit production is shown to be due to the failure of seeds to survive until appropriate temperatures permit germination. Indeed, the ecological conditions in Scotland combined with the low survival of seed in cool-moist soil before experiencing temperatures that permit germination, mainly account for the observed scarcity of bilberry seedlings (Ranwala and Naylor, 2004). Welch et al. (2000) concluded that *V. myrtillus* has a seed-dispersal strategy which secures some long-distance movement by frugivores at the expense of much lost seed, and that the small seed-bank and low numbers of seedlings result from poor defense of the seeds and weak competitive ability of the seedlings; other attributes of *V. myrtillus, e.g.* efficient vegetative reproduction, compensate for these losses and enable its success as a species.

5. Conclusions

Our work has evidenced that under proper conditions, *V. myrtillus* seeds began to germinate to a large extent without pretreatment. The optimum conditions for germination of bilberry seeds were found to be: freshly mature seeds, 16-h photoperiod, temperature of 22.5 $\pm$ 0.5 °C, use of mountain soil for *in vivo* germination or modified MS added with 9.1 μM of Zeatin for *in vitro* germination. The development of bilberry seedling was successful when a substrate of PBAS, perlite and sand was used, however the proportion of seedlings that survived after one growing season with vigorous development characterized by an adequate number of axillary shoots with sufficient elongation was a little over 50%. Hence, the true key-point is the suitability of the environment for bilberry seedling and new stand establishment.

These findings suggest that the climatic conditions of the Central Italian Apennine offer a broadened 'window of opportunity' for bilberry germination. It is probably realistic to assume that in this geographic area seedling recruitment patterns of stands include prompt and within-season germination of freshly matured bilberry seeds. Conversely, patterns observed by different authors in higher latitudes, where germination does not occur until the following spring, may explain the rarity of seedlings and the prevalence of agamic propagation by rhizomes as a strategy of *V. myrtillus* survival.

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