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Ecology and conservation strategies of target dry grassland orchid species (*Orchidaceae*)

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1. General introduction

Plant biodiversity is a fundamental component of the ecosystems. Nowadays, it is suffering a general decline, at local and global scale. The major threats for biodiversity, both at local and global scale are habitat loss and degradation due to urban and agricultural development (the primary risk for 83% of endangered plant species), unsustainable use of plant species, misuse and over-application of pesticides and fertilizers, soil erosion and contamination (heavy metals, acid rain), introduction of alien species and climate change (Sharrock and Jones, 2009).

Landscape and soil-use changes result in habitat fragmentation, in which remaining patches of natural habitat are surrounded by an anthropogenic matrix and lose structural continuity (Haila, 2002). This process not only drives species populations to size reduction and insulation but also affects the genetic quality of remnant individuals, discouraging pollinator visits and preventing gene flow through small populations (Fischer and Lindenmeyer, 2007). As a result, genetic drift and inbreeding lower the reproductive success and decrease the potential of the species to adapt to environmental changes, leading to the so-called "extinction debt", for which the actual presence of the species is no longer in balance with the present habitat characteristics and landscape configuration (Hanski and Ovaiskanen, 2002).

Moreover, the survival of some threatened species is strongly related to traditional methods of land management (coppicing, mowing, grazing) that preserved their habitat over centuries but are now increasingly forgotten. The consequent transition from the human-maintained habitats into marginal lands leads to the loss of those environmental conditions that allow endangered species to complete their biological cycle and survive (Zechmeister *et al*., 2003).

These processes could have a great negative impact on groups of highly vulnerable species that occupy narrow niche and/or have particular ecological requirements for germination, seedling establishment and reproduction (e.g. Fischer and Stöcklin, 1997; Thuiller *et al*., 2005). An example of this is offered by orchid species, since they are characterized by an high degree of ecological specialization (Pierce and Belotti, 2011) and are threatened worldwide due to habitat loss, harvesting, climate change (Knapp *et al*., 2014; Willmer, 2014; Phelps *et al*., 2015; Williams *et al*., 2015). In particular, terrestrial orchid species require high light intensities close to the ground to obtain sufficient energy for successful fruiting and thus completion of the life-cycle (Dorland and Willems, 2002; Dorland and Willems, 2006; Jacquemyn *et al*., 2008). The natural succession of vegetation and the entry of woody species can thus exclude orchids, and disturbances such as mowing interrupt succession and allow orchids to persist.

Among Magnoliophyta, *Orchidaceae* are the largest family in the world in terms of number of species (Swarts and Dixon, 2009). Two-thirds of orchid species occur in the tropics as epiphytes, with terrestrial species comprising the remaining third, yet almost half of the extinct species according to The World Conservation Union (IUCN, 1999) are terrestrial herbaceous perennials. From an ecological perspective, orchids are fascinating because of their complex life cycle, involving a vast array of reproductive variability and pollination mechanisms (Roy and Widmer, 1999), and ubiquitous interactions with mycorrhizal fungi (Burgeff, 1959). Orchids are also important from a biodiversity perspective for the large variety of life strategies among the species (Swarts and Dixon, 2009). Furthermore, some species may be locally common whilst others are extremely rare even in apparently ideal habitat (Pierce and Belotti, 2011). All these features highlight their intrinsic value as bioindicators and as research tools for defining patterns and processes that constrain how species assemble into local communities and for tuning conservation measures targeted at particularly rare species.

Conservation of orchid species is an ambitious target in most industrialized European countries due to the great loss of natural habitats and/or lack of suitable environmental conditions for these species to persist (Vogt-Schilb *et al*., 2015). Indeed, despite its richness worldwide, the *Orchidaceae* family is represented only by a small number of taxa in Europe (approximately 300 according to Delforge, 2001), all with the temperate terrestrial life form. Most of these species are reported to face alarming decline across all of Western Europe and species associated with woodlands and calcareous grasslands seem to suffer greater contractions in range than species associated with other habitats (Kull and Hutchings, 2006).

Calcareous-dry-grassland orchids in particular are threatened due to the abandonment of low-intensity agricultural regimes that maintained their habitat for centuries, but have been neglected with the spread of industrialized agriculture since the mid 1900's (Willems *et al*., 1993). The abandonment of grazing, haymaking and coppicing of woodland edges leads to a modification of dry grassland structure and functionality that impacts particularly on weakly competitive species such as orchids (Hegeduesova and Senko, 2011; Janišová *et al*., 2011).

Orchid species are also vulnerable due to their own complex life cycle which relies on the availability of non-orchid species to form symbiotic or parasitic relationships through which nutrient and pollination transfer are accomplished (Nilsson, 1992; Rasmussen, 2009). Indeed, symbiosis with mycorrhizae is pivotal to support seed germination and growth of seedlings or adult plants, yet these relationships are often species- and life stage-specific and far from being fully understood (Rasmussen, 1995; Rasmussen, 2002). Although autogamy has been reported for several orchid species (Pansarin *et al*., 2008; Bellusci *et al*., 2009; Bateman *et al*., 2015), pollen

transfer is mainly carried out by pollinating insects that are attracted by orchid flowers with different and often sophisticated mechanisms (Nilsson, 1992), which can be summarized as food rewarding, food deception and sexual deception (Schiestl, 2005).

Another important constraint to orchid conservation is posed by seed biology: orchid seeds are known to hold the record for the smallest size and weight among the *Spermatophytae* (Rasmussen, 1995), with the exception of one species (*Bletilla striata*) have no endosperm and contain few nutrient reserves (Ramsay *et al*., 1998), may have morphological and morphophysiological dormancy or particular environmental requirements for germination (Baskin and Baskin, 2014).

All of these features highlight the elevated likelihood for orchid species to be threatened, since they are characterized by many critical life stages in which their survival is at risk. Therefore it is not surprising that orchids are among the most protected plants worldwide, at least from a formal, legal point of view.

The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) of Washington (1973; *www.cites.org*), the Convention on the conservation of European wildlife and natural habitats (Bern Convention, 1979; *www.coe.int/t/dg4/cultureheritage/nature/Bern*), the Convention on Biological Diversity (CBD, 1992; *www.biodiv.org*), and the "Habitat" 92/43/EEC Directive (EU Council, 1992) are well-known cornerstones for plant species conservation policy in the European context. Nevertheless, despite the acknowledgement of an alarming and continuous biodiversity loss worldwide, current conservation actions are far from sufficient to solve the problem (Cardinale *et al*., 2012; Kanongdate *et al*., 2012).

Following the failure to achieve the "2010 Target" of the World Summit on Sustainable Development (Johannesbourg, 2002), the 10th CBD Conference of the Parties adopted the "Strategic Plan for Biodiversity 2011-2020 and the Aichi Targets" with the purpose of inspiring broad-based action in support of biodiversity over the next decade by all countries and stakeholders (UNEP, 2010). In particular the strategy addresses the reduction of direct pressures on biodiversity and the improvement of the status of biodiversity by safeguarding ecosystems, species and genetic diversity. It states that by 2020 the extinction of known threatened species has to be prevented and their conservation status has to be improved and sustained (Target 12), the traditional knowledge and practices of local communities relevant for the conservation and sustainable use of biodiversity have to be fully integrated in the implementation of the Strategic Plan (Target 18).

Because the lack of detailed information on the bio-ecology of threatened species is one of the main factors of failure of many restoration projects (Heywood and Iriondo, 2003), the Global Strategy for Plant Conservation, a CBD program, focuses attention on understanding and

documenting plant biodiversity (geographical distribution, characteristics and conservation status of species) and developing protocols for plant conservation and sustainable use (UNEP, 2011).

The need to improve scientific knowledge of species biology and ecology as an essential tool for their conservation was already established in the Habitats Directive (92/43/EEC), in the Pan-European Biological and Landscape Diversity Strategy (Council of Europe and UNEP, 2005) and, in the Italian context, in the Strategia Nazionale per la Biodiversità (MATTM, 2010).

1.1. Aim of this work

On top of the aforementioned problems, the aim of this work was to address the requirement for increasing knowledge of the biology and ecology of orchid species. Three target species, characteristic of remnant dry grasslands in the Veneto Region (NE Italy), were selected and studied, since in this highly urbanized and industrialized region, both dry-grassland orchid populations and the extension of their habitat are increasingly shrinking. Moreover, local extinction has already been reported for orchid taxa (Masin *et al*., 2006; Tasinazzo *et al*., 2007; Buffa and Lasen, 2010).
The following questions were addressed:

- What are the main structural features of the plant community that drive orchid reproductive fitness and distribution?
- Do the presence of co-flowering non-orchid entomophilous species enhance the fertilization and abundance of orchid species ?
- Which, among morphological, physical and chemical soil properties, can predict the fitness of dry grassland orchids?
- Do germination capacity of seeds from contrasting orchid populations differ due to habitat features or population characteristics?
- Is artificial outcrossing effective in enhancing germination capacity of seed from isolated orchid populations?
- Is it possible to overcome morphological dormancy in orchid seeds using “biochemical” scarification?

1.2. Target species

Target species considered in this work were *Anacamptis morio*, *Himantoglossum adriaticum* and *Ophrys sphegodes*. Nomenclature follows GIROS (2009). These species are among the most frequent dry-grassland orchid species in the study area, although consisting of clumped populations, with an estimated population density ranging from few individuals to three hundreds flowering ramets. Thus, despite the low absolute abundance of orchids, these species allowed the collection of

enough data to target the aims. Particular attention was reserved for *H. adriaticum*, since it is a European endemic species and considered of priority interest by the EU Council (92/43/CEE Annex II, 2007).

These three target orchids are sympatric species, all living in 6210* habitat [Semi-natural dry grasslands and scrubland facies on calcareous substrate (*Festuco-Brometalia*); *=important orchid sites], yet *H. adriaticum* can often be found in hedges or woodland edges. Moreover, these species differ in terms of flowering times, size and reproductive strategies. A brief description is provided below.

Anacamptis morio (L.) R.M. Bateman, Pridgeon & M.W.Chase (syn. *Orchis morio*)

This is one of the smallest dry meadow orchids, usually no more than 30 cm tall. It has a bright-green flat basal rosette made up of 5-10 narrow lanceolate leaves and a cilindrical flowering stalk baring 4-20 pink to purple (sporadically white) flowers. Two rhizotubers allow survival during the underground phase.

It is a non-rewarding orchid (food deceptive) and attracts pollinators (for the most part queen bumble bees) through the scent of the flowers and colour resembling the that of rewarding species (Smithson, 2002). It has a European-Caucasian distribution and flowering usually starts in early April in the study area.

Himantoglossum adriaticum H. Baumann

This is the largest of the dry-meadow orchid species and can be up to 1 m tall. Its inflorescence usually has 15-40 odourless flowers displaying a characteristic purple labellum resembling the shape of a lizard tongue that gives the name to the genus. Tubers are perennial organs.

Himantoglossum species are non-rewarding, since there is no evidence of nectar in the spur and they are believed to allure pollinators (wasps, bees, bumblebees, butterflies) using an unmistakable odour smelling like that of male goats, which seems to be effective also on flies and night-flying insects (Carey and Farrel, 2002).

In calcareous dry grasslands, *H. adriaticum* is the last blooming orchid before the summer drought (late May-early July) and, although it can be found in open meadows, it is often linked to a vegetation mosaic with hedges and shrub woodland edges (Kaligarič *et al*., 2004; Bòdis and Molnár, 2009). Its range comprises Italy, Austria, Slovenia, Hungary and the Czech Republic (GIROS, 2009).

Ophrys sphegodes Mill. (subsp. *sphegodes*)

This is a highly variable species in terms of floral morphology, and at least 13 subspecies have been identified in Italy (GIROS, 2009). Here we refer only to the subspecies *sphegodes*. It has a very flat basal rosette with usually one flowering stalk 10 to 50 cm high, bearing 2 to 15 flowers. Tubers are perennial organs.

This is a sexual deceptive orchid that attracts insect pollinators (Order *Hymenoptera*: *Andrena nigroaenea*, *Colletes cunicularius*, *Xilocopa iris*) thanks to the labellum shape resembling the body of the female partner (Robbirt, 2011), and a feromone-like scent that simulates the odour of female bees (Schiestl *et al*., 1997; Schiestl *et al*., 2000). It is one of the earliest blooming orchid species in continental Europe and completes its life cycle before the growth peak of the surrounding plant community (Hutchings, 1987). It has an eurimediterranean distribution, comprising South England and all of Central Europe.

2. Fine-scale drivers of dry grassland orchid distribution

2.1. Introduction

Despite *Orchidaceae* being perhaps the most diverse Angiosperm family worldwide (with 26567 species according to Joppa, 2011), only a restricted number of orchid taxa, exhibiting the temperate terrestrial life form, are found in Europe and North Africa (529 according to Delforge, 2001). These orchids have colonized a wide set of habitats ranging from the Mediterranean garigue to Arctic tundra (Dressler, 1981; Vogt-Schilb *et al*., 2015) but are particularly frequent in calcareous semi-natural dry grasslands (Barbaro et al., 2003; EU Commission, 2008; GIROS, 2009).

The presence of a rich suite of orchid species is considered by the European Commission a criterion to classify the dry grassland as an habitat of priority interest (6210*, Semi-natural and scrubland facies on calcareous substrate (*Festuco Brometalia*), EU Commission, 2007).

Different models have been suggested to explain the presence of plant species within a community as a balance between abiotic harshness, competitive and facilitative interactions among plant species (Brooker *et al.*, 2007; Vaz *et al.*, 2015; Mason *et al.*, 2011) or between plants and other organisms (Moeller 2004; Strauss and Irwin 2004). Traditionally, the rationale is based on the idea that communities assemble through a hierarchy of ecological filters (Diamond, 1975; Keddy, 1992; Weiher and Keddy, 1995; van der Maarel, 2005).

At large spatial scales, phylogeographic and historical processes select a 'regional species pool' (Ricklefs, 2004), defined as the set of species present in a region. Afterwards environmental factors (e.g. climate, land use or soil) filter adapted species from the 'regional species pool' into a 'local species pool' (Zobel, 1997). In a second stage, species from the local species pool are filtered by biotic interactions to form the 'observed communities'. Thus, while processes like dispersal limitation can initially determine which species arrive at a particular site, such processes as competition or facilitation (biotic selection), or habitat filtering processes (resource limitation and environmental gradients) will determine species persistence in a given community (Keddy, 1992; Bertness and Callaway, 1994; Lortie, 2004; Zobel and Kalamees, 2005; Gotzenberger *et al.*, 2012).

Two of the most crucial phases during these filtering and selection processes are germination and seedling establishment (Tsvuura *et al*., 2010; Jacquemyn *et al*., 2015), since seeds may have very specific ecological requirements to overcome dormancy and initiate the growth of the embryo (Baskin and Baskin, 2014) and seedlings need resources and a protected environment to resist abiotic harshness, herbivory or parasitic attacks and become adult plants (Eriksson, 1995; Moles and Westoby, 2004). Thus, at the local scale, the availability of favourable sites for a

permanent establishment of juvenile plants is essential for a species to overcome the selection and persist within the community (Clark, 1999).

Several of those factors have been taken into account to explain the distribution of orchid species in dry-grasslands, with particular attention for climate conditions (Wotavová *et al.,* 2004), nutrient availability (Silvetown *et al.,* 1994), physic environment (Kull *et al.,* 2006) and anthropogenic disturbance such as fire and grazing (Hutchings, 1998; Gregg, 2004; Coates *et al*., 2006; Janišová *et al.,* 2011).

However, there is little knowledge about the relationship between orchid distribution and the structural attributes of surrounding vegetation (Landi *et al.,* 2009), despite such attributes being of pivotal importance in semi-natural grasslands where they are mainly determined by traditional human management practices as moving, pasturing, haymaking (Janišová *et al.,* 2011). European Union Habitat's Directive (Directive 92/43/CE) habitat number 6210 in particular is characterized by the presence of scrubland facies, which may become dominant on grassland patches in case of abandonment, with the development of thermophile fringe vegetation (*Trifolio-Geranietea*) and thermophile scrub (EU Commission, 2007). In the latter case, the permanence of orchid species would be seriously threatened due to colonization by woody species (Jersáková *et al*., 2002; GIROS, 2009). Therefore it should be expected that changes in vegetation structure between managed grassland and abandoned scrubland patches could impact the distribution of orchid species.

Within this framework, the aim of this study was to assess the relative importance of community structural constraints on the distribution and reproductive fitness of three orchid species (*Ophrys sphegodes*, *Anacamptis morio* and *Himantoglossum adriaticum*) that are relatively widespread between dry grasslands and nearby ecotonal mesoxeric scrubland/woodland patches in the Veneto Region.

2.2. Methods

Study area

The study took place on three hill massifs of the Veneto Region, NE Italy: the Eastern Lessini Mounts, Berici Hills, and the Euganean Hills Regional Park (NE Italy, 45°20'-45°30'N, 11°25'- 11°45'E; Fig.1). These localities were designated as Special Protction Areas (SPA) and Sites of Community Interest (SCI) according to Directive 92/43/EEC, as hosting a rich suite of endangered or endemic species and habitats of community interest. In particular the area encloses, within a few square kilometres, 5% of the regional dry grassland surface, which hosts 14 orchid species (Rizzieri Masin and Tietto, 2006): *Ophrys apifera* Huds., *Ophrys sphegodes* Mill., *Ophrys*

holoserica N. L. Burman, *Ophrys benacensis* (Reisisgl) O. & E. Danesch & F. Ehrend; *Serapias vomeracea* (Burm.) Briq., *Himantoglossum adriaticum* H. Baumann, *Anacamptis morio* (L.) R.M. Bateman, Pridgeon & M.W.Chase, *Anacamptis pyramidalis* (L.) L. C. Rich, *Orchis papilionacea* L., *Orchis tridentata* Scop., *Orchis purpurea* Huds., *Orchis militaris* L., *Orchis simia* Lam. and Spiranthes spiralis (L.) Koch.

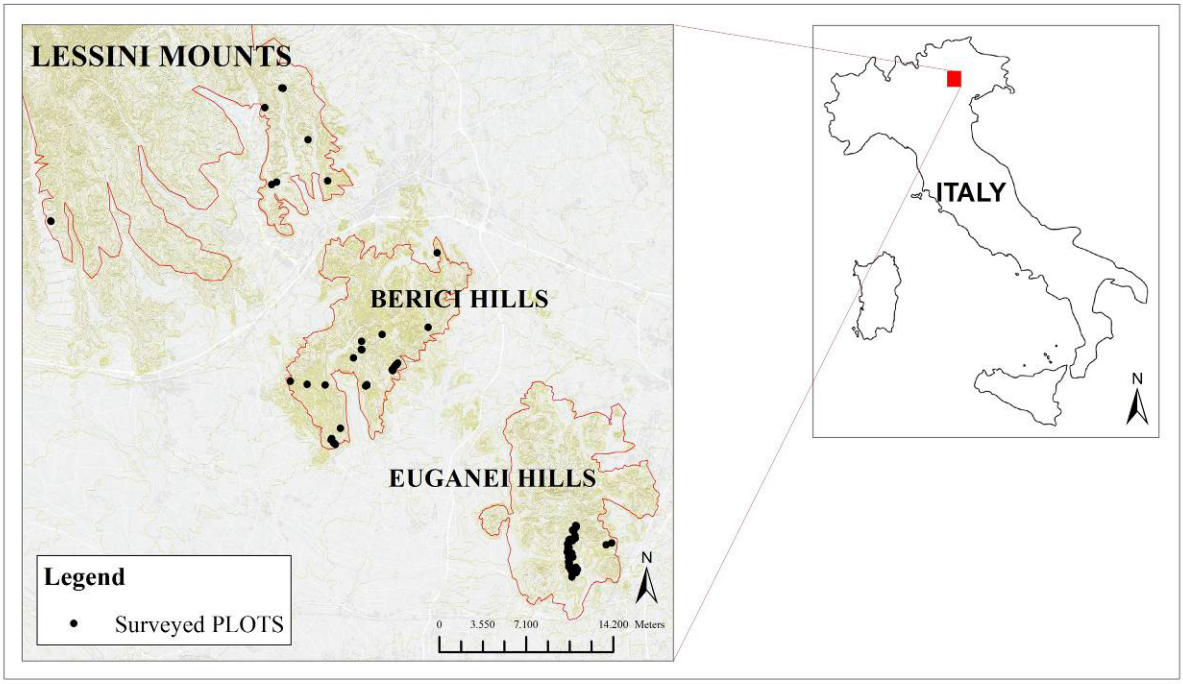

Figure 1: Study area and distribution of the surveyed plots

The area encompasses several low-altitude hills with a major peak at 602 m a.s.l., originating both from sedimentary (limestone and marl) bedrock and volcanic (basaltic and riolitic) formations from the end of the upper Eocene, completely isolated from the nearest massifs and surrounded by the alluvial Venetian Plain (the Easternmost part of the Po Plain).

Local climate data (*http://www.arpa.veneto.it/bollettini/htm/dati_idrometeo.asp*) reveal an average annual rainfall from 850 to 1200 mm, with two maximum peaks in April and September and two minimum peaks in July and December. A drought season is generally absent (Tomasi *et al.,* 2011), however, calcareous mountainsides covered by dry grasslands are characterized by a very low soil available water content (AWC), leading to a noticeable edaphic aridity from late spring to late summer (Bini, 2001). Mean annual temperature in the lowland is 13°C, with a peak mean high temperature of 23.5°C in July and a low of 3°C in January. Therefore, according to Rivas-Martínez (2005) the bioclimate can be classified into temperate oceanic, with an upper mesotemperate thermotype, lower subhumid ombrotype.

Study sites were represented mostly by small to medium-sized (0.2-2 Ha) dry grasslands, dispersed in an agricultural landscape among forests and arable fields. According to the local Corine Land Cover 2006 classification (*http://idt.regione.veneto.it//app/metacatalog/*), the prevalent land use categories are pastures and agriculture (57%, including annual and permanent crops, olive groves and vineyards) followed by broad-leaved forests with transitional woodland-shrub areas (41%) and discontinuous urban fabric and road network (2%). Dry grasslands develop on middle-altitude limestone slopes (47-443 m a.s.l.), on sites characterised by thin and primeval soil (Inceptisols and Entisols), with average pH of 7.5 (Bini, 2001).

Once exploited for haymaking or cattle grazing, dry grasslands experienced increasing abandonment until the institution of the Park and the SPA/SCI management plan, according to which they are partly subjected to management such as mowing and grazing (with a frequency of once a year to once every three years).

Data collection

Vegetation was sampled during spring–summer (April–June) in 2013 using both a preferential (on target orchid populations) and a stratified random sampling to select plots within the boundaries of dry grassland patches of the land use cartography of the Veneto Region (2009). The risk of spatial autocorrelation between plots was minimized by fixing an average distance between plots greater than 270 metres, which allows to consider plots and data herein collected as spatially independent (Rezende *et al.*, 2015). Altogether 92 2 m × 2 m plots were collected, resulting in 53 plots with the presence of target orchid species, and 39 without.

In each plot, all plant species were recorded and the projected cover of all species was visually estimated using a percentage scale. Total percentage vegetation cover (hereafter "TotCov"), total percentage of shrub and tree species cover ("WCov") and total percentage cover of herbaceous species ("HerbCov") were visually estimated as the projection of the entire vegetative canopy or herbaceous layer canopy on the ground.

Moreover, the mean herbaceous vegetation height ("HerbH") was estimated as a by-cover-weighted average of the height of single species, measured with a rigid ruler from the ground surface to the highest point of the ramet (regardless whether it was a vegetative part or inflorescence). The height of every single species was measured on 5 ramets, when present, or on all ramets in case of low-frequency species.

For the three target orchid species, *Anacamptis morio, Himantoglossum adriaticum* and *Ophrys sphegodes*, population size data were also estimated as total percent cover of rosettes ("OrcCov") within each plot. Moreover, for 203 tagged individuals (59 for *A. morio*, 52 for *H.*

adriticum and 92 for *O. sphegodes*) the following plant traits were recorded: height of the flowering stalk, number of flowers and number of fruits. The number of tagged individuals per plot represented at least the 5% of the local density of flowering ramets (number of flowering stalks/ 4 m^2 plot) and, for very small populations (local density of flowering ramets ≤5) it coincided with the number of flowering ramets. Data were collected at the peak of species flowering season following the protocols described in Cornelissen *et al*. (2003).

A vegetation database referring to the 92 plots was built in TurboVeg 2.114 (Hennekens, 2014). As synthetic descriptors of the community structure for each plot, the species richness ("N", as the total number of vascular species) was determined, and Pielou's evenness index (J) was calculated to take into account the distribution of abundance across species (Mulder *et al*., 2004). These parameters were calculated using the diversity index editing tool provided in TurboVeg 2.114.

Additionally, to analyze the effect of vegetation structure on the target species, reproductive fitness was estimated at the individual level using the fruit/flower ratio ("FFr") of marked ramets. The relative inflorescence height of each ramet (RH = flowering stalk height – mean herbaceous vegetation height) was then calculated as a synthetic variable describing the structural relationship between target species' ramets and the closest surrounding vegetation. RH has a positive value when the flowering stalk is taller than the surrounding vegetation, and is otherwise negative (specifically the herbaceous vegetation overtops the entire above-ground shoot of the orchid).
The variables used in this work are summarized in Table 1.

Table 1: Definitions of the variables and relative abbreviations used in this work

Abbreviation	Meaning
OrcCov	total percent cover of orchid rosettes in the plot
J	Pielou's evenness index
N	total vascular species richness in the plot
TotCov	total percent vegetation cover in the plot
WCov	total percent shrub and tree vegetation cover in the plot
HerbCov	total percent herbaceous vegetation cover in the plot
HerbH	mean height of herbaceous vegetation in the plot
RH	relative inflorescence height of each ramet (RH = flowering stalk height – mean vegetation height)
FFr	fruit/flower ratio of marked ramets

Data analysis

Species richness (N), evenness index (J), total vegetation cover (TotCov), shrub and tree species cover (WCov), herbaceous species cover (HerbCov), and mean herbaceous vegetation height were selected as predictors of the abundance of orchid species at the plot level.

To quantify the effect of these predictor variables on the abundance of target orchid populations, a generalized multiple regression model (GLZ) was performed on plot data considering orchid cover (OrcCov) as the covariate and species richness (N), evenness index (J), vegetation cover (TorCov; WCov and HerbCov) and herbaceous vegetation height (HerbH) as independent variables. The use of a GLZ model was necessary due to the high frequency of low values for the covariate and thus a non-normal distribution even following commonly used data transformations.

The analyses were performed using the Generalized Linear Model module of Statistica 8.0 software (StatSoft Inc., 2008) selecting a Poisson data distribution and a Log link function due to the assumption that the effects of the predictors on the covariate (OrcCov) are not linear and unlikely to occur for minimal variations of the independent variables (McCullagh and Nelder, 1989).

To assess the effect of vegetation structure on orchid reproductive fitness, a Regression Model was built for each target species in Statistica 8.0 using the FFr as the covariate and relative ramet height (RH) as the predictor.

2.3. Results

The surveyed plots exhibited a very rich cumulative species pool (249 species were counted overall), with a mean species richness (N) of 24 (±5.41 SD) per plot, ranging from a minimum of 10 to a maximum of 41.

According to the evenness index (J), species were relatively equally distributed in the majority of surveyed plots, with an average value of 0.72 (±0.11 SD). The highest values of J (up to 0.92) were found in plots with highest N dominated by tussocks while lowest values (0.32 to 0.5) were found in the most abiotically harsh locations largely dominated by dwarf shrubs (*sensu* Cornelissen *et al*., 2003).

TotCov and HerbCov were generally high (82.3 ±15.53 % and 72.3 ±15.46 %, respectively), with few exceptions on eroded mountainsides where the plot surfaces were characterised by up to 40% of bare ground. On the contrary, shrub and three species cover was generally low (6.8 ±13.45%), although exceeding 25% in 10 plots with target orchids. HerbH ranged from 10 cm to 1m, with a mean value of 34.7 cm (±14.70 SD).

GLZ models (Table 2) revealed that HerbCov and HerbH had a significant effect on the dependent variable (OrcCov), with a negative trend (Fig. 2), while N, J and TotCov had no

significant effects. As for the relative importance in the model building, HerbH was the major contributor (χ^2=17.1), although HerbCov played a comparable role (χ^2=14.62).

Table 2: Results for Generalized Regression Model (Poisson distribution, Log Link Function) on orchid cover (OrcCov): β=regression coefficients estimates, SE=standard errors of β, Wald and p_W =Wald statistics and p-values for the significance of β, Log-lik=log-likelihood for the model that includes the effects of the given variables and all others before it, χ2 and p_{LRT} = incremental χ2 statistic and relative p-value. Abbreviations: N=number of vascular plant species, J=Pielou's evenness index, TotVegCov=total vegetation Cover, HerbCov=herbaceous vegetation cover; HerbH= mean herbaceous vegetation height. Marked values are significant at α≤0.05.

	β	SE	Wald	pW	Log-lik	χ2	pLRT
Intercept	1.452	0.6631	4.799	0.028	-213.2		
TotCov	0.001	0.0054	0.085	0.771	-211.6	3.19	0.074
WCov	-0.005	0.0062	0.539	0.463	-211.1	0.40	0.524
HerbCov	**-0.012**	**0.0051**	**6.228**	**0.013**	**-204.2**	**14.62**	**<0.0001**
N	0.018	0.0162	1.33	0.249	-202.6	3.31	0.069
J	0.754	0.8004	0.89	0.346	-202.1	0.98	0.322
HerbH	**-0.025**	**0.0072**	**14.078**	**<0.0001**	**-193.5**	**17.10**	**<0.0001**

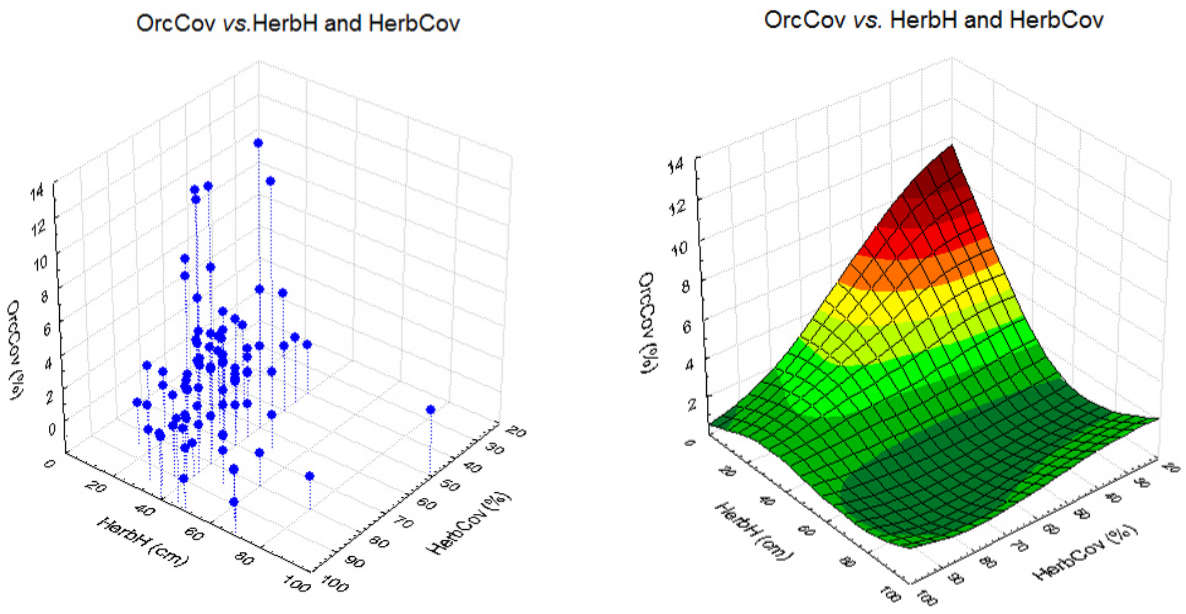

Figure 2: Orchid cover (OrcCov) as a function of herbaceous vegetation height (HerH) and cover (HerbCov). On the left: 3D scatterplot; on the right: surface plot (Distance Weighted Least Squares).

Regression models on FFr (Table 3) revealed that the proportion of fertilized flowers in the wild was very significantly affected by RH both for *Ophrys sphegodes* (β=0.435, p_β<0.001; Fig. 3) and *Himantoglossum adriaticum* (β=0.88, p_β<0.001; Fig. 5). In particular, a very high closeness of fit was obtained for *Himantoglossum adriaticum* (Adj R^2=0.771).

On the contrary, such a relationship was not evident for *Anacamptis morio* (F=0.0003, p_F <0.987; Fig. 4).

Table 3: Results of regression models between the fruit/flower ratio (FFr) and the relative inflorescence height (RH). Abbreviations: β=regression coefficients estimates, SE=standard errors of β, t_β and p_β= t Student statistics and p-values for the significance of β, Adj R^2= R^2 corrected for the regression model; F and p_F = Fisher statistics and associated p-value for the significance of the model, n=number of cases (marked ramets).

Species		β	SE	t_β	p_β	Adj R^2	F	p_F	n
Ophrys sphegodes	Intercept			9.066	<0.0001				
	RH	0.435	0.095	4.577	<0.0001	0.179	20.95	<0.0001	92
Anacamptis morio	Intercept			5.464	<0.0001				
	RH	0.002	0.132	0.016	0.988	0	0.0003	0.987	59
Himantoglossum adriaticum	Intercept			18.795	<0.0001				
	RH	0.880	0.067	13.128	<0.0001	0.771	172.35	<0.0001	52

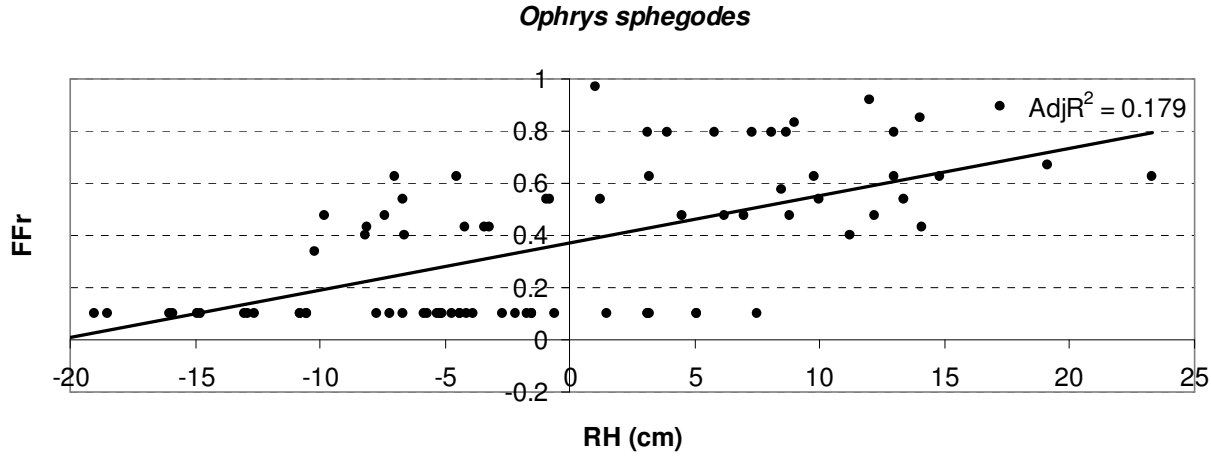

Figure 3: Scatterplot of *O. sphegodes* fruit/flower ratio (FFr) *vs*. relative inflorescence height (RH)

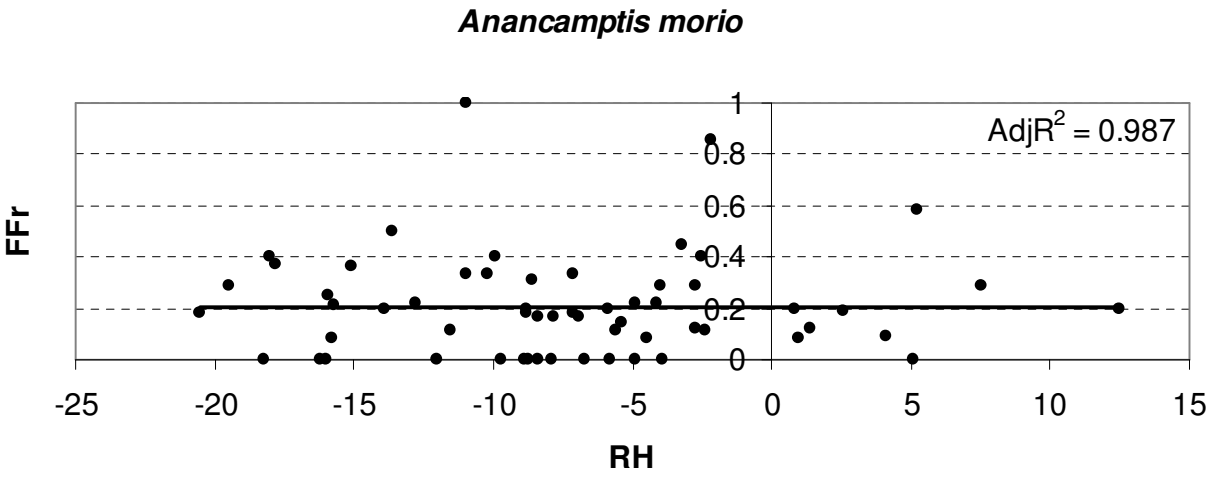

Figure 4: Scatterplot of *A. morio* fruit/flower ratio (FFr) *vs*. relative inflorescence height (RH)

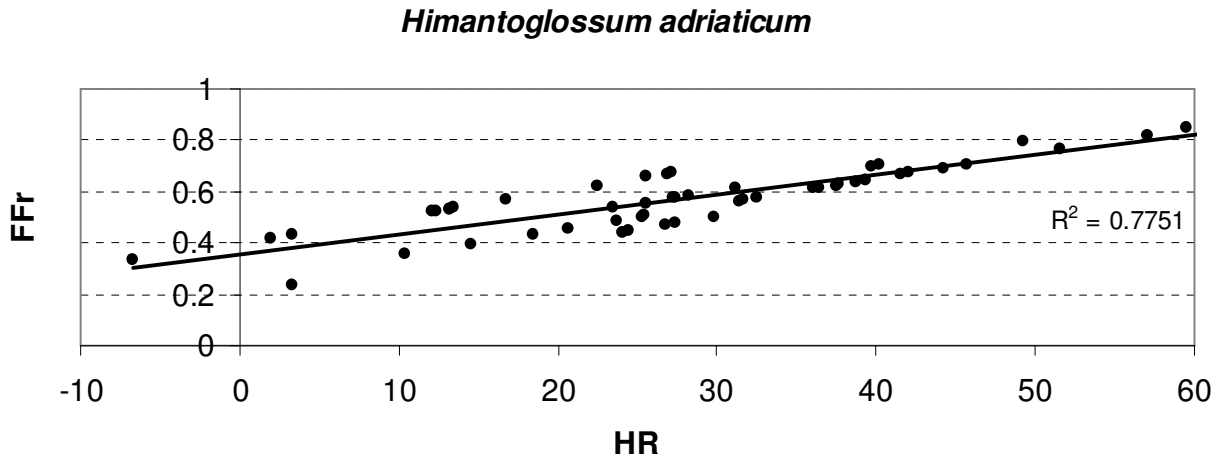

Figure 5: Scatterplot of *H. adriaticum* fruit/flower ratio (FFr) *vs*. relative inflorescence height (RH)

2.4. Discussion

The distribution pattern of target dry grassland orchid species appears to be driven by the physical structure of the herbaceous vegetation, both on the horizontal and vertical plane, while species richness, evenness, total cover and cover of shrub and tree species did not affect target orchid abundance.

Orchid populations exhibited the maximum cover in plots with larger portions of bare ground and low herbaceous vegetation, regardless of the total cover. On the one hand, this finding appears to support the theory of microsite limitation (Clark, 1999), according to which the most important abiotic constraint to plant establishment is the availability of micro-habitats on the soil surface suitable for seed germination and seedling establishment (Jacquemyn *et al.*, 2007a).

On the other hand, it is counter-intuitive that total vegetation cover or cover of shrub and tree species did not play a role in regulating orchid population abundance, but it must be noted that the flowering periods of *O. sphegodes* and *A. morio* pre-empt the peak of vegetative development of the woody species. Moreover, the majority of *O. sphegodes* and *A. morio* plots had a low shrub and tree cover (2.8±7.36%) in comparison with herbaceous cover (65.3±13.12%), and thus it could be argued that woody species did not affect orchid abundance simply because they were sporadic in the surveyed plots.

H. adriaticum is characterized by a wider ecological plasticity than *O. sphegodes* and *A. morio*, since this species was found both on open dry grasslands and under tree canopies, with a woody species cover ranging from 0 to 90%. Nevertheless, *H. adriaticum* cover is not determined by the extent of the woody species canopy, since populations with extreme cover values ranging from 0.5 to 6% were found in contrasting vegetation types. It is known that this species often lives in a woodland/scrubland/dry grassland mosaic (Bodis and Molnar, 2009), but it is still unclear

whether this is due to precise ecological requirements, such as shelter from frost and predation (Bodis and Botta-Dukat, 2008) or specific fungal symbionts (Pecoraro, 2013) or is the result of habitat transformation (invasion by scrubland or woodland) after the abandonment of traditional management practices in former open dry grassland (Bodis and Molnar, 2009). However, despite the fact that it may be present under a tree canopy, *H. adriaticum* was consistently found very close to open areas and it was never found in a continuous woodland understory.

Additionally, the neutral role of shrub and tree species cover for target orchid distribution could be due to the result of two counteracting effects. On one hand, woody species colonization of dry grassland is the consequence of their abandonment and transition to scrublands or woodlands where grassland orchids would be overcome by most competing species and lose their regeneration niche (Poorter, 2007; Santiago *et al*., 2013; Zhang *et al*., 2014). On the other hand, while the grassland/scrubland mosaic still exists, woody species might actually favour the persistence of grassland orchid species living in the ecotonal areas, offering shelter from extreme drought and heat, predation and excessively early spring mowing (Bertness and Callaway, 1994; Armas *et al*., 2011; Ziffer-Berger *et al*., 2014).

At the ramet level, vegetation height had a very strong effect on the fruit set of *O. sphegodes* and *H. adriaticum* in accordance with their respective ramet heights: in those individuals that were able to raise their inflorescence over the surrounding vegetation, the fruit set was richer. This suggests a greater visitation rate by pollinators to the tallest inflorescences, perhaps due to a greater visual attraction.

It is known that the structure of vegetation surrounding orchid populations could select for those ramets that are able to attract pollinators on their inflorescences and in this sense limits pollinator availability only to ramets bearing visible floral displays (Kindlmann and Jersáková, 2006). Pollinator attraction and thus female reproductive success in plants generally increases with greater floral display size (Grindeland *et al*., 2005), but for strongly pollinator-limited deceptive orchids there is some evidence that the main trait targeted by this pollinator-mediated selection is the inflorescence height (e.g.: Sletvold and Agren, 2011, Walsh *et al*., 2014).

Therefore, it could be hypothesized a pollinator-mediated selection on flowering stalk height for *O. sphegodes* and *H. adriaticum,* but such a selection would be relative to the surrounding herbaceous vegetation height and not absolute (namely, it may not act on the flowering stalk height *per se*). These findings are of great interest with respect to those of Sletvold *et al*. (2013, 2014): they demonstrated that pollinator-mediated selection for taller plants is stronger in tall vegetation for the food deceptive orchid *Dactylorhiza lapponica* but is not active in short vegetation and is weaker for the rewarding orchid *Gymnadenia conopsea*.

Pollinator-mediated selection on inflorescence height of sexual deceptive orchid was supposed by Peakal and Handel (1993) for the Australian orchid *Chiloglottis trilabra*, whose pollinators discriminated among floral heights in a choice experiment, although they did not find evidence in the field due to very low numbers of inflorescences of wild populations. An increase in pollination due to greater plant height was found also for American food deceptive *Cypripedium* species (O'Connel and Johnson, 1998; Wake, 2007).

On the contrary, according to our results, it seems such a selection is lacking on flowering stalk height for *A. morio*, since fruit set was not affected by relative ramet height. There are two main reasons that could explain this: a greater intrinsic visibility of its inflorescence and reduced vegetation height. The deep purple inflorescence of *A. morio* could have much more attractive power toward pollinators with respect to the brownish or white flowers of the other two target orchids that blend into the tussocks, since darker colours are supposed to be more tempting to pollinating bees than weak colours (Willimer, 2011). As for the second reason, mean herbaceous vegetation height in *A. morio* plots was just 25 cm and such a low value may depress the expression of pollinator-mediated selection (Sletvold *et al.,* 2014) because it is almost equal to the mean flowering stalk height (24.5±5.4 cm).

Despite high species richness being perhaps the most evident feature of dry grasslands, the distribution of target orchid species within this habitat was not sensitive to richness or to the evenness index. Such a finding corroborates the hypothesis that the main driver of orchid distribution in the study habitat is the microsite availability for plant establishment rather than a suite of species-specific relationships with non-orchid species or, whether any of these relationships were important, they would arise between orchids and restricted gild of species but not at the community level.

In conclusion, this work demonstrate that, at the study scale (4 m^2), these particular orchid species are very sensitive to herbaceous vegetation structure rather than to the structure of the whole plant community. This implies a major role of interactions between orchids and more competitive herbaceous species (graminoids in particular), while a scarce cover of woody species seems not to affect the distribution of target orchids.

Since the regulation of vegetation height in the study area is determined by human management (no wildfires, strong snowfalls or other significant natural events occur), maintaining all those disturbance regimes linked to traditional practices in the most appropriate period of the year (late summer, or in any case after the dispersal of seeds, Janeckova *et al*., 2006) must be considered of primary importance to maintain the proper structure and functionality of dry grasslands (Janisova *et al*., 2011).

Indeed, long-term low-intensity agriculture systems with mid-summer mowing or grazing and reduced fertilizer application are a common feature of most well-preserved species-rich grasslands in Europe (e.g.: Maccherini, 2006; Rolecek *et al*., 2014), allowing the maximum expression of coexistence mechanisms between grassland species (Wilson *et al*., 2012).

3. Effect of co-flowering species on orchid pollination and distribution

3.1. Introduction

The assemblage of species in plant communities is a current topic of debate in ecology. Two of the main theories that are currently used to explain the coexistence of species within a community: the dispersal-assembly model, based on stochastic processes (MacArthur and Wilson, 1967) and the niche-assembly model, based on ecological filters determined by local abiotic conditions or biotic selection (Chase and Leibold, 2003).

Abiotic constraints to plant life select for those genotypes bearing physiological tolerance and filter out the least suited (Keddy, 1992), yet possibly the plant community itself modifies the physical environment at the micro-scale (e.g. light intensity beneath the plant canopy, soil moisture, ground temperature; Bertness and Callaway, 1994; Tsvuura *et al*., 2010).

Biotic selection refers to all intra- and inter-specific relationships, either positive or negative, responsible for the selection of those species able to withstand competition with other plant species, to take advantage from them (i.e. selection due to facilitation mechanisms) or successfully resist parasites or animal attack (Wotavova *et al*., 2004; Gotzenberger *et al.,* 2012).

In particular, interactions among plants for pollination seem especially fascinating since pollinators are generally considered a resource for which plants compete (Rathcke, 1983; Pierce *et al.,* 2007). As such, competition among plants for pollinator services can determine the floral community structure through processes such as competitive exclusion and differentiation of floral forms or phenologies (Campbell, 1985; Stone *et al*., 1998; Fishman and Wyatt, 1999; Caruso, 2000). Alternatively, it has been proposed that co-flowering plants may, instead of competing, actually facilitate pollination (Brown and Kodric-Brown, 1979; Waser and Real, 1979; Rathcke, 1983; Callaway, 1995) enhancing pollinator visits due to increased floral display, thus allowing pollen transfer also in smaller, isolated ramets, or in those hidden by surrounding vegetation (Elzinga *et al*., 2007).

Inter-specific interactions with co-occurring plant species could be of pivotal importance for pollination services for many orchid species, particularly for fertilization of deceptive ones. Indeed orchids are known so far for their peculiar pollination strategies (Sprengel, 1793; Darwin, 1885; Pouyanne, 1917): approximately one third of orchid species rely on food deception and hundreds of species use sexual deception (Nilsson, 1992; Steiner *et al*., 1994). With the food deception strategy, orchid species effectively steal pollinators from nectar-rewarding neighbouring species by mimicking their floral attractants (Schiestl, 2005), while orchid species that use sexual deception

lure pollinators while they are foraging in a patch of rewarding plants by using visual and olfactory baits resembling mating signals of the same insect species (Cozzolino and Widmer, 2005).

Therefore it can be argued that deceptive orchids should in turn rely on pollinator sharing processes with co-flowering non-orchid species that provide nectar and support pollinator activity before they are deceived by orchid themselves (Cozzolino and Widmer, 2005).

Moreover food deceptive orchids that profit from co-flowering non-orchid magnet species for pollinator availability should provide the same floral signals provided by rewarding plant species to their respective pollinators in order to be chosen by deceived insects (Lammi and Kuitunen, 1995; Smithson and Macnair, 1997; Jersáková *et al*., 2006). Since the greater part of temperate terrestrial orchid species are pollinated by a limited array of pollinator families (Nilsson, 1992; Van der Cingel, 2001; GIROS 2009), it would imply a convergence in the floral forms between orchid and non orchid species to make the pollinator sharing effective (Internicola *et al.*, 2007). In particular, the reproductive fitness of deceptive orchids seems to increase when their flower colour resembles that of co-flowering rewarding species (i.e. "non-model mimicry", Dafni, 1984).

Nevertheless, although deceptive strategies are thought to rely on pollinator sharing with rewarding species, very few studies have been devoted to understand the role of non-orchid entomophilous companion species on orchid fitness (e.g.: Johnson *et al*., 2003, Pellegrino *et al*., 2008).

Within this framework, the aim of this study was to evaluate the role of non-orchid entomophilous plant species in governing the flower pollination and distribution of three target dry-meadow orchid species: *Anacamptis morio*, *Ophrys sphegodes* and *Himantoglossum adriaticum*. In particular flowering synchrony was postulated as a necessary pre-condition of a possible pollinator sharing between target orchids and non-orchid species.

The hypothesis were: a) there is a non-random flowering pattern between orchid and non-orchid species; b) pollination rate of orchid flowers is positively affected by flowering time overlapping and the abundance of co-flowering species displaying the same flower colour of orchids; c) orchid abundance increases as synchrony increases; d) there is a convergence in floral forms between orchid and non-orchid co-flowering species.

3.2. Methods

Study area

The study took place in the Regional Park of Euganean Hills (NE Italy, 45°N, 11°E; Fig. 1). The area, protected by a regional law since 1989, has been also designed as SPA and SCI according to the Directive 92/43/EEC, for hosting a rich suite of endangered or endemic species and habitats of community interest. Among these, are "*Semi-natural dry grasslands and scrubland facies on*

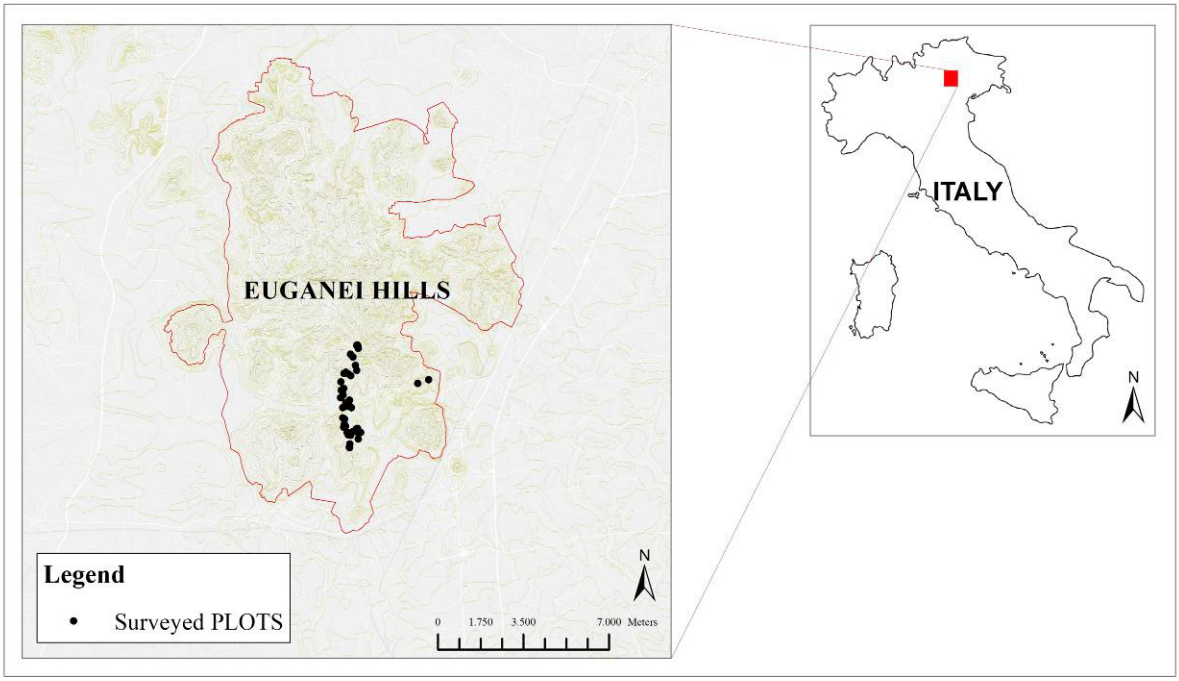

Figure 1: Study area and distribution of the surveyed plots

calcareous substrates (Festuco-Brometalia)" (*important orchid site, EU Commission, 2007) which occupy 13% of the Park area (AA. VV., 2006).

The area, of 22000 hectares, is composed of several low-altitude hills (250 m. a.s.l. on average) of sedimentary origin (Jurassic limestone and Low Eocenic marl) in the South and volcanic origin (Upper Eocenic basaltic lavas and Oligocenic riolites) in the North, completely isolated from the nearest mountain range (Alps) by the alluvial Venetian Plain (Astolfi and Colombara, 1990).

Climatic features are intermediate between Continental and Mediterranean ones, with strong micro-climatic differences due to the local geomorphology (aspect and slope). Mean daily temperatures range from winter lows of 3°C to summer highs of 24° and average annual rainfall is of 850 mm, with two maximum peaks in April and October and a winter minimum in January (historical data available at *http://www.arpa.veneto.it/bollettini/htm/dati_idrometeo.asp*).

Bioclimate can therefore be classified into temperate oceanic, with an upper mesotemperate thermotype, lower subhumid ombrotype (Rivas-Martinez, 2005). Although a summer drought season is generally absent due to term convective precipitation (Chiaudani, 2008), a significant edaphic aridity is expected from late May to the beginning of September due to very low soil water content on calcareous mountainsides with thin primeval soils (Bini, 2001).

In this particular situation, dry-grasslands have established and been maintained by traditional human management until a few decades ago, when drastic changes in the local social-economical pattern triggered the abandonment of mowing, haymaking and pasturing practices. As a consequence, dry-grasslands were reduced in size due to invasion from scrubland or woodland, and populations of species herein, particularly the most sensitive as orchids are, underwent drastic losses (AA.VV., 2006). In recent years, the institution of the management plan by the Park authorities has guaranteed more effective conservation policies and nowadays the area still represents a significant hotspot of dry-meadow orchid diversity (Rizzieri Masin and Tietto, 2006).

Data collection

Vegetation was sampled during spring–summer (April–June) in 2013 using a preferential sampling method targeted to orchid populations. Altogether 53 2×2 m plots were collected, resulting in 13 plots referring to *A. morio*, 20 to *O. sphegodes* and 20 to *H. adriaticum*. In each plot, all entomophilous plant species were recorded and the projected cover of these species was visually estimated by using a percentage scale. Species nomenclature followed Conti *et al*. (2007).

During the flowering season of dry-meadow orchids in 2013 (April, 1st – June, 10th), in each plot the flowering phenology of all entomophilous species was surveyed. Flowering time has been recorded, every ten days, collecting binary data on flowering occurrence (0=no flowers at all, 1=flowering ongoing). Flowering was considered to have started when the first flower opened on an individual plant and terminated when the last anther was lost, as in Dante *et al*. (2013).

For the three orchid species the population density within a plot was quantified as the rosette percent cover (OrcCov, see Table 1 for a description of the abbreviations). Moreover, on 203 marked individuals, the number of flowers and number of fruits were collected to calculate the fruit/flower ratio (FFr, see Chapter 2). Data were collected at the peak of target orchid flowering season following the protocols described in Cornelissen *et al*. (2003).

Data analysis

Overlapping in flowering time was evaluated using a co-flowering index (V coefficient; Lepš and Šmilauer, 2003) on binary (0=absence of flowering; 1=flowering ongoing) species × time intervals matrices. V-coefficients, also called V-score (V_{sc}) are very synthetic indexes that allow quantification of the species aggregation or segregation and range from -1 (maximum separation between flowering periods) to 1 (two species flowering on exactly the same days for the entire flowering season).

To test for an overall aggregated or segregated pattern in flowering time, a "Frequency" Null Model Test (Gotelli and Graves, 1996) was applied with 999 random permutations (Kembel *et al*., 2010; R Core Team, 2015). Subsequently, flowering synchrony was quantified at the plot and community (grassland) level as the mean value of all V_{sc} referring to every pair of orchid and non-orchid entomophilous species found in a single plot (PV_{sc}) or in all 50 plots respectively (MV_{sc}).

A mean fruit/flower ratio (FFR) was calculated for each plot using all FFr per ramets available in a given plot. This derived variable was considered a proxy of the pollinator visitation rate to flowers at the plot level.

Two generalized regression models (GLZ) were built to assess the role of the independent variable (PV_{sc}) on the covariates (FFR and OrcCov). The analysis were performed using the Generalized Linear Model module of Statistica 8.0 software (StatSoft Inc., 2008) with a Poisson distribution and a Log link function for the OrcCov data model, a Normal distribution and Log link function for FFR data models.

To test for the effect of co-blooming rewarding species flower colour on the orchid pollination rate, all the entomophilous species with a synchronous flowering with the target orchids (*A. morio*, *H. adriaticum or O. sphegodes*) where classified according to the colour of their respective flowers/inflorescences in three categories: PURPLE (sharing the same colour of *A. morio* inflorescence), WHITE (sharing the same colour of *H. adriaticum* inflorescence) and YELLOW (sharing the same colour of *O. sphegodes* inflorescence). Such a classification was performed using the information about flower colours available in the Biolflor database (Kuhn *et al*., 2004). The total cover of all species belonging to each class was calculated and considered as the predictors of orchid FFR. Three distinct GLZ models were built to asses the role of "purple-", "white-" and "yellow-" flower species cover on *A. morio*, *H. adriaticum* and *O. sphegodes* FFR, respectively, as the covariates.

To evaluate the convergence in floral morphological traits between orchid and co-flowering non-orchid species, for each plot all entomophilous species were grouped into four categories of

distinct flower structures, following the "blossom types" classification of Kugler (1970) available in the Biolflor database (Kuhn *et al*., 2004). Categories were named as follows:

- Zygomorphic (ZYG), strongly zygomorphic flowers with a closed corolla, including orchid species;
- Disk (DSK), actinomorphic flowers with an open corolla;
- Head (HED), pseudanthium-type inflorescences;
- Bell-Funnel-Tube (BFT), actinomorphic flowers with a closed corolla.

The total percent cover of species grouped in each category was computed and used as a community functional parameter (Violle et al. 2007) expressing quantitatively the relative importance of the different blossom types within the entomophilous plant community.

Generalized regression models were built to assess the effect of the total percent cover of the four blossom type groups (ZYG, DSK, HED, BFT) on PV_{sc}. The analysis were performed using the Generalized Linear Model module of Statistica 8.0 with a Poisson distribution and a Log link function, both for the three target species altogether as *Orchidaceae* group and for the single target orchids.

Table 1: Brief explanation of all the abbreviations used in this work

Abbreviation	Meaning
OrcCov	total percent cover of orchid rosettes
V_{sc}	V-score (flowering time overlapping index) between two entomophilous species
PV_{sc}	mean value of all V_{sc} between orchid and non-orchid species computed at the plot level
MV_{sc}	mean value of all V_{sc} referring to every pair of entomophilous species found in the study area dry-meadows (50 plots)
FFr	fruit/flower ratio of a tagged ramet
FFR	average value of FFr among all tagged ramets within a plot
ZYG	total percent cover of species group of zygomorphic blossom type
DSK	total percent cover of species group of disk blossom type
HED	total percent cover of species group of head blossom type
BFT	total percent cover of species group of head and bell-funnel-tubular blossom type

3.3. Results

The mean number of entomophilous species recorded per plot was 12.8 (±3.7 SD), ranging from a minimum of 3 to a maximum of 22. The most common species were *Bupleurum baldense* Turra*, Cerastium pumilum* Curtis, *Convolvulus cantabrica* L., *Crepis sancta* (L.) Babc, *Globularia bisnagarica* L.*, Helianthemum nummularium* (L.) Miller, *Medicago minima* (L.) Bartal., *Scabiosa triandra* L., *Sonchus asper* (L.) Hill and *Thlaspi perfoliatum* L. (mean frequency 57.5%), though their abundances were very low (mean cover values per plot between 0.3 and 3.4%). The most

abundant species were *Spartium junceum* L., *Geranium sanguineum* L., *Galium lucidum* All. and *Onobrychis arenaria* (Kit.) DC. (mean cover values per plot between 4.8 and 8.8%) though they were quite rare (mean frequency 12.5%).

The total cover of flower colour classes was higher for the WHITE class (8.8±6.3%), followed by the YELLOW class (6.6±4.5%) and PURPLE (2.9±2.4%).

The total cover value of blossom type groups was generally higher for the ZYG group (43.5±28.5%), followed by DSK (31.3±24.2%), HED (15±21.7%) and BFT (10.2±17.2%).

Target orchids flowered for a short period (no more than 25 days). Among them, *O. sphegodes* and *A. morio* were among the earliest species to initiate flowering in the Euganean dry grasslands: the *O. sphegodes* flowering period lasted from the first to the third week of April; the *A. morio* flowering period lasted from the second to the fourth week of April. However, *H. adriaticum* flowered in the middle of dry grassland flowering peak, namely from the third week of May to the second week of June.

The flowering synchrony pattern proved to be significant both at the plot level ($0.0786<PV_{sc}<0.3581$; $0.001<$Null model $p<0.046$) and community (dry grassland) level ($MV_{sc}=0.141$; Null model $p=0.0008$), and an amount of 147 significant synchronous species-specific relationships were found between target orchids and companion species. The most frequent non-orchid species that co-flowered with orchids were *Cerastium pumilum* Curtis and *Cerastium brachypetalum* Desp ex Pers. (DSK group, 18.4% of cases both), *Globularia bisnagarica* L. (BFT group, 8.2%), *Euphorbia falcata* L. (DSK group, 7.5%), *Crepis sancta* (L.) Babc (HED group, 6.8%) and *Thlaspi perfoliatum* (DSK group, 4.8%).

GLZ models on FFR and OrcCov revealed that PV_{sc} had no significant effects on the dependent variables considered (Table 2, Fig. 2). Moreover, the total cover of co-flowering species classified according to the purple, white and yellow flower colour proved not to be a predictor of the target orchid FFR (Table 3).

GLZ models on PV_{sc} showed that none of the four blossom types cover was a predictor of the flowering synchrony between orchid and non-orchid species, both for the *Orchidaceae* group altogether and single target orchid species (Table 4, Fig.3). Thus, neither a convergence nor a divergence in floral forms with respect to flowering synchrony of co-occurring dry grassland species were found.

Table 2: Results for GLZ models on orchid fruit/flower ratio (FFR) and cover (OrcCov) for A). Fruit/flower ratio, B). Orchid cover: β=regression coefficients estimates, SE=standard errors of β, Wald and pW =Wald statistics and p-values for the significance of β, Log-lik=log-likelihood for the model that includes the effects of the given variables and all others before it, $\chi 2$ and p_{LRT} = incremental $\chi 2$ statistic and relative p-value. PV_{sc} =mean V-score a the the plot level.

A. Fruit/flower ratio (Normal distribution, Log link function)

	β	SE	Wald	pW	Log-lik	$\chi 2$	p_{LRT}
Intercept	4.56	1.025	19.859	0.000	-112.634		
PV_{sc}	-0.29	1.056	0.079	0.779	-112.143	0.092	0.762

B.Orchid cover (Poisson distribution, Log link function)

	β	SE	Wald	pW	Log-lik	$\chi 2$	p_{LRT}
Intercept	0.08	0.631	0.016	0.900	-60.286		
PV_{sc}	1.16	0.857	1.834	0.176	-58.175	1.836	0.175

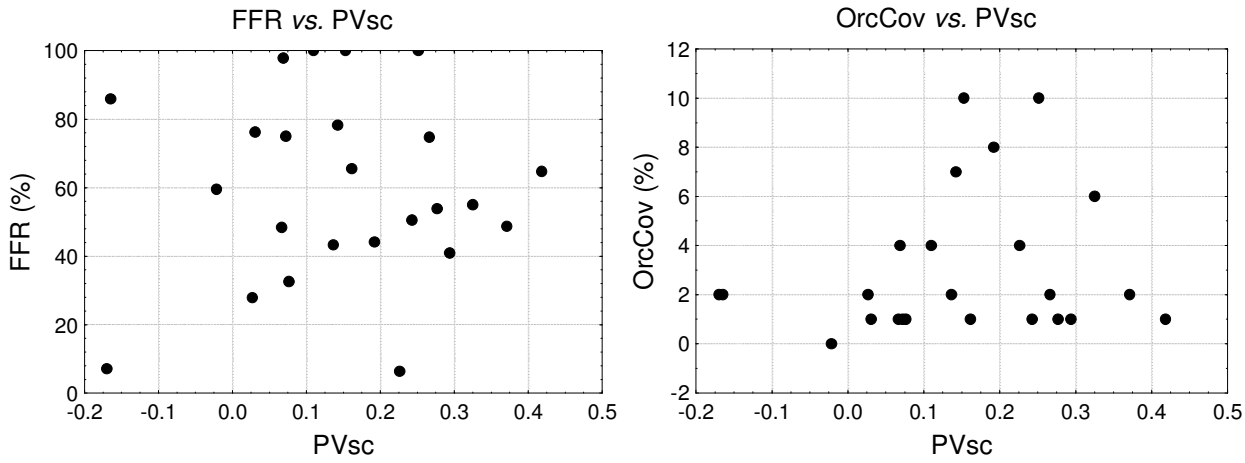

Figure 2: Scatterplots of Frut/flower ratio (FFR, on the left) and orchid cover (OrcCov, on the right) as a function of the mean V-score at the plot level (PVsc).

Table 3: Results for GLZ models on orchid fruit/flower ratio (FFR) using the total cover of all the entomophilous species co-flowering with the target orchid and grouped according to the orchid flower colour. A). *A. morio* FFR *vs.* total cover of species in the "PURPLE" flower class, B). *H. adriaticum* FFR *vs.* total cover of species in the "WHITE" flower class, C). *O. sphegodes* FFR *vs.* total cover of species in the "YELLOW" flower class. β=regression coefficients estimates, SE=standard errors of β, Wald and pW =Wald statistics and p-values for the significance of β, Log-lik=log-likelihood for the model that includes the effects of the given variables and all others before it, $\chi 2$ and p_{LRT} = incremental $\chi 2$ statistic and relative p-value. PV_{sc} =mean V-score a the the plot level.

Model	Variable	β	SE	Wald	pW	Log-lik	$\chi 2$	pLRT
A	Intercept	-1.59	1.207	1.74	0.187	-3.732		
	PURPLE	0.01	0.266	0.00	0.982	-3.732	0.001	0.982
B	Intercept	-1.40	1.129	1.53	0.216	-5.034		
	WHITE	0.01	0.100	0.01	0.907	-5.027	0.014	0.907
C	Intercept	-3.01	2.172	1.92	0.166	-4.217		
	YELLOW	0.12	0.205	0.36	0.551	-4.035	0.365	0.546

Table 4: Results for GLZ models (Poisson distribution, Log link function) on PV_{sc} (A=all orchid species; B=*A. morio*; C=*H. adriaticum*; D=*O. sphegodes*), : β=regression coefficients estimates, SE=standard errors of β, Wald and pW =Wald statistics and p-values for the significance of β, Log-lik=log-likelihood for the model that includes the effects of the given variables and all others before it, $\chi 2$ and p_{LRT} = incremental $\chi 2$ statistic and relative p-value. ZYG, DSK, HED, BFT=total percent cover of species groups referring to the zygomorphic, disk, head and bell-funnel-tubular blossom types.

A. All orchid species

	β	SE	Wald	pW	Log-lik	$\chi 2$	p_{LRT}
ZYG	-0.37	8.041	0.002	0.963	-11.527	0.004	0.952
DSK	2.97	6.249	0.227	0.634	-11.446	0.164	0.686
HED	1.71	6.652	0.066	0.797	-11.410	0.072	0.789
BFT	3.74	7.177	0.272	0.602	-11.301	0.217	0.641
Intercept	-2.19	0.914	5.720	0.017	-11.529		

B. *Anacamptis morio*

	β	SE	Wald	pW	Log-lik	$\chi 2$	p_{LRT}
ZYG	5.79	17.741	0.107	0.744	-3.329	0.001	0.978
DSK	-44.18	92.385	0.229	0.632	-3.205	0.248	0.618
HED	37.42	309.775	0.015	0.904	-3.198	0.015	0.903
BFT	-100.94	176.649	0.327	0.568	-2.964	0.468	0.494
Intercept	-0.89	1.535	0.337	0.562	-3.330		

C. *Himantoglossum adriaticum*

	β	SE	Wald	pW	Log-lik	$\chi 2$	p_{LRT}
ZYG	9.35	36.771	0.065	0.799	-3.725	0.004	0.952
DSK	-3.44	28.963	0.014	0.905	-3.720	0.008	0.927
HED	17.96	58.739	0.094	0.760	-3.550	0.340	0.560
BFT	1.93	11.468	0.028	0.866	-3.536	0.028	0.867
Intercept	-3.01	3.389	0.784	0.376	-3.726		

D. *Ophrys sphegodes*

	β	SE	Wald	pW	Log-lik	$\chi 2$	p_{LRT}
ZYG	-4.47	18.358	0.059	0.808	-4.438	0.019	0.891
DSK	7.25	10.553	0.472	0.492	-4.262	0.352	0.553
HED	4.68	9.794	0.228	0.633	-4.175	0.174	0.677
BFT	16.57	69.851	0.056	0.813	-4.149	0.053	0.819
Intercept	-2.69	1.942	1.927	0.165	-4.447		

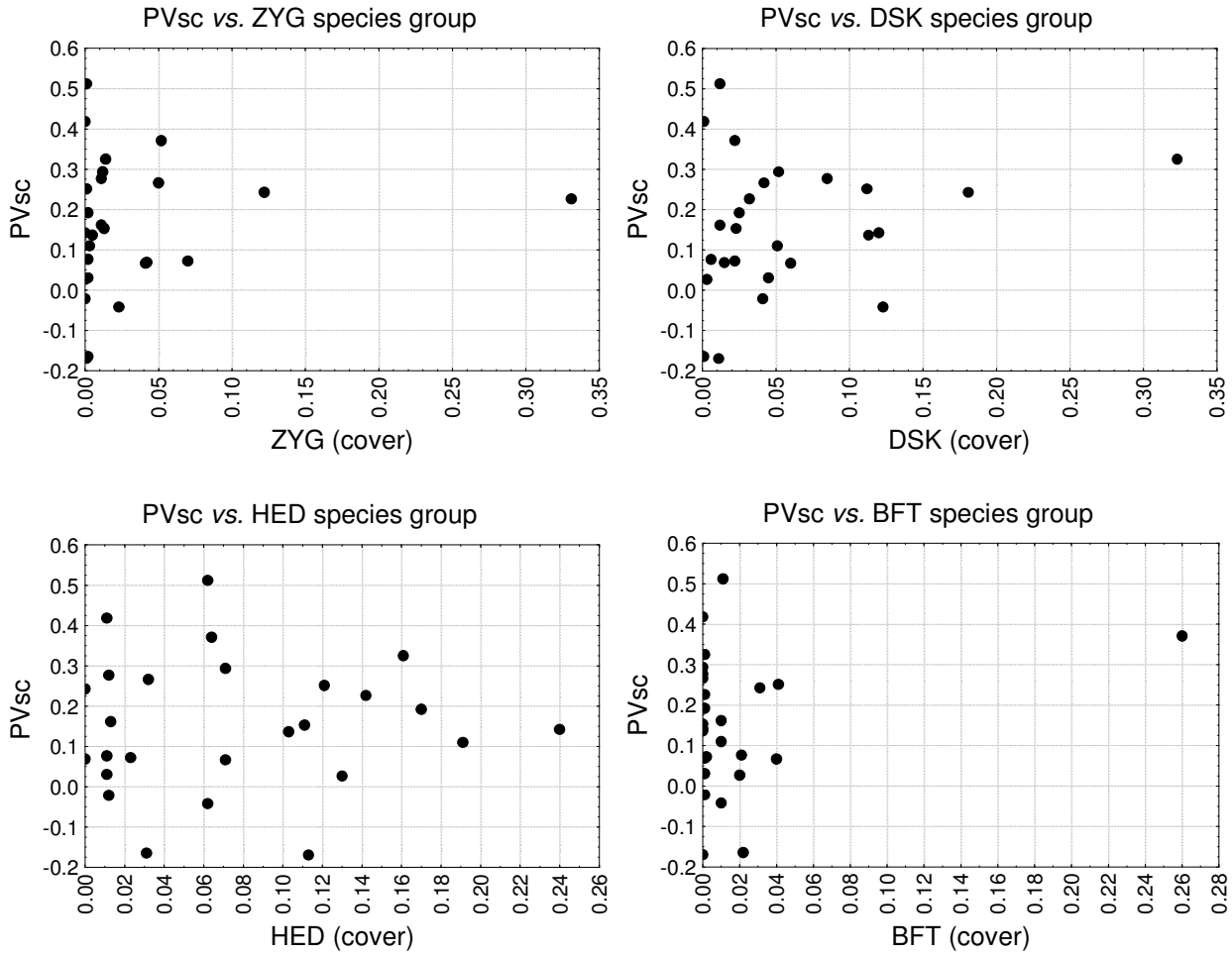

Figure 3. Scatterplots of mean V-score at the plot level (PVsc) as a function of the cover of all the entomophilous species classified into the "Zygomorphic" (ZYG) group (up left), "Disk" (DSK) group (up right), "Head" (HED) group (down left) and "Bell-Funnel-Tube" (BFT) group (down right). Cover value are in a 0-1 scale (1=100%).

3.4. Discussion

Mean V score at the plot level (PV_{sc}) was hypothesized to have a positive effect on target species fruit/flower ratio and orchid cover (hypothesis "b"), since it is diagnostic of a shared flowering period between the orchids and the enthomophilous community and thus it is a premise of possible pollinator sharing, the target species being adapted to attract pollinators through deceptive mechanisms while they are visiting non-orchid species (Ayasse *et al.,* 2000; Carey and Farrel, 2002; Schiestl, 2005).

In this study, pollinator sharing between orchid and non-orchid species could be inferred in case higher values of fruit/flower ratio and PV_{sc} had been found in plot with higher cover of "zygomorphic" species (i.e. a convergence in floral traits between co-flowering species). Alternatively, lower values of fruit/flower ratio and PV_{sc} in presence of relevant cover of zygomorphic species would indicate a competition for pollination services.

Contrary to expectations, and despite a significant synchronous flowering pattern both at the grassland and plot scale (validating hypothesis "a"), PV_{sc} turned out not to be a predictor of either fruit/flower ratio or of orchid cover. This outcome could be explained with the distribution of synchronous non-orchid species, mostly belonging to the genera *Cerastium, Globularia, Crepis* and *Thlaspi,* which are among the most frequent but also less abundant species, often with cover values less than 1%. Such a low abundance could also explain the absence of an effect on the orchid pollination rate (FFR) from the total cover of the entomophilous species co-flowering with orchids and grouped by flower colour. Therefore, it could be argued that a non-model mimicry is unlikely to occur in this case due to a weak abundance of the rewarding plants. Moreover, among the co-flowering species, none belongs to the same blossom type of orchid species ("Zygomorphic"), since most of them were classified into "Disk" and "Head" groups. This implies both a weak floral display of co-flowering species and a very negligible capability as magnet species toward orchid-pollinating insects. Only *Spartium junceum* and *Onobrychis arenaria* present the same morphological floral traits of orchids (zygomorphic flowers with a closed corolla), yet, despite a greater floral display with respect to frequent synchronous species, their occurrence was limited to a few plots, and thus the likelihood of deterministic pairwise species interactions was necessarily low, particularly in high-diversity communities such as dry-grasslands (Myers and Harms, 2011).

The absence of a convergence or divergence in floral forms between orchids (both as a group and as single target species) and co-flowering species suggests that the binary relationships between these two groups of sympatric species are somewhat neutral with respect to pollinator sharing.

According to these outcomes, the hypothesis that flowering synchrony is an enhancer of orchid fitness (hypothesis "b" and "c") due to a convergence in floral forms between orchid and co-flowering non-orchid species (hypothesis "d"), as a necessary precondition for the pollinator attraction by magnet species (Thomson, 1978, Johnson et al., 2003), must be rejected.

These findings are actually more prone to support the "remote habitat hypothesis" (Lammi and Kuitunen, 1995; Gumber and Kunze, 2001), according to which food deceptive orchids would obtain higher pollination success in times and at places where few other rewarding species are flowering. This mechanisms would be targeted at exploiting few inexperienced pollinators visiting a low-density flowered habitat rather than deceiving pollinators while foraging high-density rewarding species, since insects learn to avoid rewardless plants after only a small number of visits, and favour nectariferous species (Ferdy *et al*., 1998; Internicola *et al*., 2008).

Such an explanation would be especially pertinent for *A.morio*, since it is one of the earliest-flowering species in the study area (the second week of April) which pre-empts the flowering peak of most part of co-occurring dry grassland species. This anticipation has also been recorded in British populations of *A. morio* and interpreted as an adaptation to avoid summer heath and drought, using essentially a Mediterranean life cycle (Wells *et al*., 1998). Moreover it could be viewed as a strategy to avoid competition with co-occurring rewarding species (and simultaneously to avoid vegetative competition for light; Pierce *et al*., 2014) rather than to benefit from a pollinator sharing. Indeed, early-flowering of non-rewarding species is supposedly an adaptive strategy allowing exploitation of the relative abundance of naïve pollinator at the beginning of flowering season and/or limit the competition for pollinators with later-flowering rewarding species (Pellissier *et al*., 2010).

However, this hypothesis cannot be applied to *H. adriaticum*, since it is a late flowering orchid (third week of May). Its pollination strategy is rather unclear, even though there is no evidence of nectar production in co-generic *H. hircinum* and the closely-related *Barlia robertiana* (Carey and Farrell, 2002). Therefore, neutral relationships regarding access to pollinators between *H. adriaticum* and co-flowering species are the most plausible scenario according to our data. In Chapter 2 a strong evidence was found about the relative height of *H. adriaticum* flowering stalks as an important driver of flower fertilization rate. This suggest that structural attributes of the inflorescence (rather than the morphology of single flowers) are more effective cues to effectively attract pollinators. In other words, *H. adriaticum* could attract pollinators directly, rather than exploiting other magnet species (it could be a magnet species itself). As seen for other larger-leaved terrestrial orchids, such as *Anacamptis pyramidalis*, the large size of *H. adriaticum* is likely to allow growth and flowering even when the surrounding dominant species are starting to attain height

during spring, when the smaller orchids such as *A. morio* are already overshadowed and cannot grow (Pierce *et al*., 2014).

For *O. sphegodes* the explanation could rely on the extreme specialization of this orchid, a sexually deceptive species. This species is known to deceive males of solitary bees of the genus *Andrena* by releasing volatile compounds that resemble the female pheromones (Schiestl *et al*., 1997) and pollination is ensured only by one or a small number of species. Indeed it has been demonstrated that geographically-distinct Thyrrenian and Adriatic populations establish an almost exclusive relationship with *Andrena bimaculata* and *A. nigroaena*, respectively (Breitkopf *et al*., 2013). Floral scent seems to be the leading signal to specific pollinator attraction in *O. sphegodes*, whereas floral display appears to be a secondary cue (Vereecken and Schiestl, 2009; Ayasse *et al*., 2011). Since *Andrena* males usually patrol non-rewarding sites for mating (Schiestl and Ayasse, 2000), it appears unlikely that non-orchid co-flowering species, even those of the same blossom type of orchids, can share foraging *Andrena* bees with orchid species.

Nevertheless, it is worth noting that the negligible effect of flowering synchrony at the plot scale could become important at higher scales (larger plots or entire grasslands), since the facilitative interactions by sympatric species are scale-sensitive. Dante *et al*. (2013) found that flowering synchrony was significantly more than expected by chance in 1 m^2 plots, yet Johnson et al. (2003) proved that pollinator visitation rate to flowers of *A. morio* was significantly positively correlated with the density of sympatric co-flowering *Geum rivale* and *Allium schoenoprasum* in a 100 m^2 patch or at the grassland (up to 2 ha) level, but not in 1 m^2 patches.

Several studies (Wilson *et al.,* 1987; Stoll and Weiner, 2000*;* Watkins and Wilson, 2003) suggest that inter-specific interactions mostly act at small spatial scales, their effect being overshadowed by the role of habitat filtering or ecological variability at larger scales. Nevertheless, it has been reported that local history and dispersal limitation may be more important factors in regulating the target species distribution rather than actual facilitative mechanisms (Zobel, 1997; Lortie *et al*. 2004). In other words, as niche differentiation in dry grasslands is limited due to severe abiotic conditions, the past land use, local extinction or migration phenomena and stochastic seed dispersal could weaken the impact of positive biotic inter-specific relationships (Zobel and Kalamees, 2005; Zobel and Partel, 2008).

Although the debate regarding whether stochastic (*sensu* Diamond, 1975) or deterministic (*sensu* Clements, 1916) forces prevail in driving ecological community composition is still an open issue, different processes are widely recognised as the shaping agents of plant communities: historically contingent events, species-specific adaptations to local abiotic features, competitive-facilitative interactions among individual plants and interaction with non-plant organisms (Lortie, 2004).

This study was aimed at evaluating the relative contribution of entomophilous co-flowering species on the fertilization and abundance of target dry-grassland orchids. Possible facilitative relationships (enhanced pollinator sharing via co-flowering non-orchid sympatric) species were taken into account and modelled to quantify their importance in regulating orchid fruit/flower ratio and cover, but no effects at all were detected for flowering synchrony index (co-flowering V-score).

These findings demonstrate that, at the study scale ($4m^2$), target orchid species are not sensitive to co-flowering patterns with non-orchid species. However, the field of facilitative interactions for pollination services is very complex and little understood (Willimer, 2011), and thus further investigation of the role of flowering synchrony and subsequent pollinator sharing are required at different scale levels to get a better understanding of its impact on target species conservation.

Ultimately, given the results of Chapter 2, it could be argued that the main biotic selection on target orchids is operated, rather than by interactions between co-flowering species for pollinator access, by the entire herbaceous species community due to competition for resources, that impact on floral display, in turn influencing the flower fertilization rate and orchid cover.

4. Effects of soil features on *Himantoglossum adriaticum* fitness

4.1. Introduction

Environmental features and resource limitations, such as water, temperature, light, space and nutrient availability, are very important in determining the community assemblage and plant diversity pattern (van der Maarel, 2005; Lomba *et al*., 2011). At the local scale, these feature are the main drivers of plant species fitness (Keddy, 1992; Lortie, 2004; Zobel and Kalamees, 2005).

Moreover, geomorphological heterogeneity has proven to overlay habitat characteristics, affecting resource availability and energy-mass dynamics (water infiltration/runoff, incident solar irradiance, mineral weathering, erosion/deposition cycles), soil features (texture, chemical properties) and climatic conditions, even at the micro-scale (Brancaleoni *et al*., 2003; Stallins, 2006; Dana and Mota, 2006; Kim and Yu, 2009).

Soil properties are important constraints to plant life since they determine the availability of a wide range of resources. As for physical factors, soil depth, abundance of gravel or stones, texture and organic matter content influence the volume of air and water retention capacity which are key factors for plant physiology (Bruckner, 1997; Orozco et al., 1997, Giordano, 1999). As for chemical factors, the underlying bedrock composition, subsequent weathering phenomena, accumulation or depletion of organic matter and nutrients dynamics determine pH and macro- and micro-nutrient content availabilities to which plant species are often strictly adapted (Mauseth, 1998).

The edaphic environment is apparently of pivotal importance for orchid germination and establishment (Ors *et al*., 2011), since orchid seeds are extremely reduced in size, lack endosperm tissue and thus have very little reserve substances for growth and development, are likely to suffer from desiccation or runoff and rely on a mycorrhizal association for nutrient uptake (Rasmussen, 1995).

Although the morphology and biology of orchid species has received much more attention (Schlegel et al., 1989), at present, few studies have considered the relationship between soil properties and orchid fitness and in most cases have specifically investigated the edaphic environment associated with micorrhizal symbionts (e.g.: Lu *et al*., 2012; Bunch *et al*., 2013; Jacquemyn *et al*., 2015). Seed germination of the terrestrial orchid *Neottia ovata* was reported to be highly correlated with soil moisture and pH, while nutrients (NH_4^+, NO_3^-, P) and organic carbon had no effect (Jacquemyn *et al*., 2015). Survival of orchid protocorms is generally hindered by inorganic nitrogen and phosphorous supply in asymbiotic culture (Mead and Bulard, 1979; Arditti, 1992). The use of nitrate and phosphate fertilizers in the wild has been shown to have adverse

consequences on biomass and flowering rate of *Anacamptis morio* and *Dactylorhiza fuchsii* populations (Silvertown *et al*., 1994; McKendrick, 1996).

The present study aimed to test whether soil physical-chemical and morphological variables influence the fitness of *H. adriaticum* and to identify which of these are the most relevant predictors. We decided to focus on *H. adriaticum* since it is a CITES species and it is listed in the Annex II of the Habitat Directive 92/43/CEE, as a species of European priority interest. However, very little information is available regarding its ecological requirements with respect to soil properties. It is known that *H. adriaticum* develops on calcareous or dolomitic bedrock, both on grasslands and scrubland or woodland mosaics (Bodis and Botta-Dukat, 2008; Bodis and Molnar, 2009; GIROS, 2009), but detailed information on the role of soil properties on the development and fitness of this species are still lacking.

4.2. Methods

Study Area

The study took place in three hills massifs of the Veneto Region, NE Italy: Eastern Lessini Mounts, Berici Hills, Euganean Hills Regional Park (NE Italy, 45°20'-45°30'N, 11°25'- 11°45'E; Fig.1), where *H. adriaticum* populations grow only on limestone soils at low-altitude (50-400 m a.s.l.) in an homogeneous bioclimatic area (mesotemperate subhumid oceanic according to Rivas-Martinez, 2005).

These soils have been exploited since the Roman colonization (II Century B.C.) due to their favourable position and local climatic conditions and used for agriculture, pastures or human settlements (AA.VV., 2006). This exploitation has favoured the erosion of the topsoil and produced a regressive evolution which reduced the original depth and altered the natural soil profile.

Thus, according to USDA Soil Taxonomy (Soil Survey Stuff, 1999), most calcareous soils in the study area can be classified as Eutrochrepts, and are shallow (even less than 40cm in eroded areas), contain abundant gravel and have a low available water capacity. The soil profile is elementary, with a thin topsoil (the A horizon is no more than 20 cm deep), a weathered subsoil (Bw horizon) ending in the bedrock. These soils are characterized by high cationic exchange capacity and high content of $CaCO_3$, which confers a sub-alkaline pH (Bini, 2001).

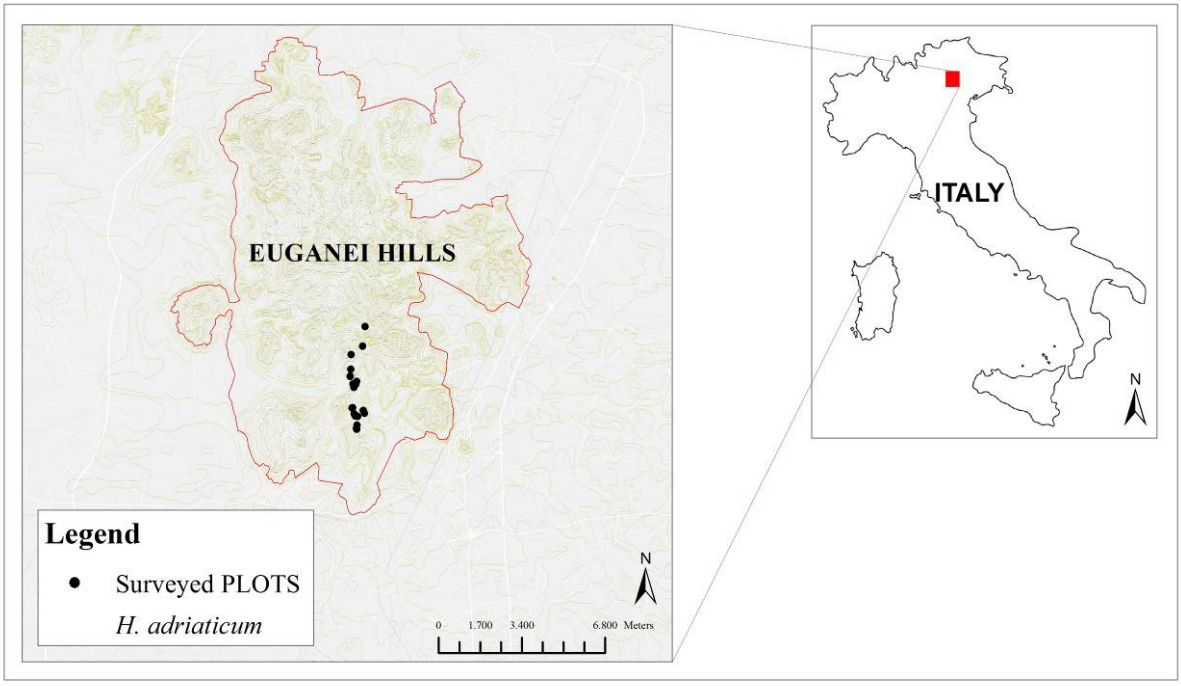

Figure 1: Study area and distribution of the surveyed plots

Data collection

Twenty populations of *H. adriaticum* were sampled at the peak of flowering in May 2014 on 2×2 m plots. As a proxy of population size, the density of all ramets (OrcDen, for a list of abbreviations see Table 1) found in the plot were used (i.e., number of orchid ramets/4 m^2).

Then the following plant traits were measured for 6 to 10 marked ramets, for each plot: leaf length and leaf width of all the leaves of a tagged ramet, height of flowering stalk (FSH) (Cornelissen *et al*., 2003).

To account for geomorphic variability, in each plot topographic data were collected: aspect (AS), expressed in degrees clockwise from the North and slope (SL), measured in percentage steepness with respect to the horizontal plane (steepness=0%).

Soil samples were made up by four subsamples of equal mass collected in the four corners of each plot in September, 2014. As a subsample reference area, a 20 cm-diameter circle was used and subsamples referring to a given plot were carefully mixed together just after collection. Every subsample included, where present, Organic (O) and Topsoil (A) horizons, with a depth of 8 to 15 cm (depending on local features affecting soil profile structure such as slope and amount of rocks). Vegetation and litter were removed to ensure that only well-decomposed organic matter was incorporated with the topsoil in the final sample. All rocky material found on the soil surface or partially buried was kept to determine gravel and stone content. Only O and A horizons were considered because the greater part of biological activity in the soil occurs here and the hypogeous

parts of orchid ramets (tubers and roots) are found within the first 15 cm under the ground surface (Jacquemyn *et al*., 2015).

On average, 9 kg of soil and gravel were collected for each plot. Soil samples were transferred to lab facilities just after collection and stored in dark polyethylene bags at ambient conditions for no more than two days prior to analysis.

Soil analysis

Soil samples were air dried for 72 h at 24°C and 50% RH and soil aggregates were subsequently broken using a ceramic mortar. Roots and partially decomposed organic residues were removed from the samples. All rocky pieces greater than 7.5 cm in diameter were weighed separately (Kern precision balance 440-49A, Balingen, Germany) to determine the stoniness (SS). Ground samples were sieved with 2mm-mesh inox woven-wire sieve (Endecotts, London, UK) and sieve content was weighed to determine the percentage of gravel (GR).

The fraction of soil particles <2 mm was then used for the determination of the following physical properties (texture classes according to USDA; Soil Survey Division Staff, 1993) and chemical parameters:

- Percentage of clay (CL; diameter of particles < 2µm) [method II.5];
- Percentage of silt (ST; 2µm<diameter of particles<50µm) [method II.5] ;
- Percentage of sand (SN; 50µm<diameter of particles<2mm) [method II.5];
- Content of nitric nitrogen ($N\text{-}NO_3^-$; mg/Kg) [method IV.2; DR2010 protocol 8192];
- Content of orthophosphates (PO_4^{3-}; mg/Kg) [method IV.2; DR2010 protocol 8178];
- Content of carbonates (CO_3^{2-}; g/Kg) [method V.1];
- Content of organic carbon (CO; g/Kg) [method VII.3];
- pH [method III]

The information in square brackets indicates the analytic methods used, described in Ministerial Decree 25/03/2002, Italian Ministry of Agriculture/Ministero delle Politiche Agricole e Forestali and, when used, the number of protocol of DR2010 Spectrophotometer Procedures Manual, Hach Company, 2000.

Data analysis

Leaf length and leaf width were used to estimate the leaf area of every measured leaf using the simple formula Leaf Area = (Leaf Length · Width)/2 that proven to be effective for the elliptic-lanceolate leafs of terrestrial orchids (Janekova *et al*., 2006). Leaf Area values of each ramet were added together to obtain the total plant leaf area, and these values were averaged within a plot to

obtain a mean plant leaf area (PPLA). Height of flowering stalks (FSH) were averaged within a plot as well (PFSH). Mean plant leaf area, mean height of flowering stalks and ramet density were chosen as dependent variables explaining the fitness of *H. adriaticum* populations.

The local topographic features for each plot were summarized with a "topographic" index, that took into account the Southness Index (SI=180-|180-AS|; Chang et al. 2004) and the slope (SL):

$AI=SI \cdot SL$

where AS=aspect, i.e. angular distance from North in degrees, and SI was converted in a 0-100% scale before multiplication. Since the index accounts for soil surface inclination and exposure to the sun, it also accounts indirectly for the runoff and amount of solar radiation per unit surface. It was thus named "Aridity Index" (AI) and theoretically ranges from 0 (completely North-facing mountainsides with very-gentle slopes or completely flat areas) up to 9000 (completely South-facing mountainsides with 90° slopes).

All soil physical variables (SS, GR, CL, ST, SN) chemical variables ($N\text{-}NO_3^-$,PO_4^{3-}, CO_3^{2-}, CO, pH) and the Aridity Index were tested as possible predictors of *H. adriaticum* vegetative fitness.

To find a structure in the relationships between these variables and select the most appropriate independent variables explaining the fitness covariates, a Principal Component Analysis (PCA) was carried out in Statistica 8 using the Multivariate Exploratory Technique tool (StatSoft Inc, 2007). To meet the requirement for PCA, data were log or arcsin-square root transformed when necessary. Soil predictors were than selected according to the correlation coefficient (factor loading) between variables and the first two principal components, using a factor loading threshold of ±0.7.

Before to proceed with further analyses, the correlation of the selected variables was tested and three distinct Generalized Regression Models (GLZ) were built for PPLA, PFSH, OrcDen (as covariates) and the uncorrelated soil predictors.

Table 1: Description of abbreviations used for variables

Abbreviation	Meaning
OrcDen	orchid population density, measured as number of ramets/4m2
PPLA	mean total plant leaf area at the plot level (cm2)
PFSH	mean height of flowering stalks at the plot level (cm)
AI	Aridity Index = SI · SL (Southness Index · SLope, dimensionless)
SS	Stoniness (%) = (weight of rocky pieces >7.5 cm)/(weight of bare soil sample)
GR	Gravel content (%) = (weight of rocky pieces <7.5 cm and >2 mm)/(weight of bare soil sample)
CL	clay content (%)
ST	silt content (%)
SN	sand content (%)
$N\text{-}NO_3^-$	nitric nitrogen (mg/Kg)
PO_4^{3-}	orthoposphates (mg/Kg)
CO_3^{2-}	carbonates (g/Kg)
CO	organic carbon (g/Kg)

4.3. Results

All fitness covariates (Table 1, Annex 1) varied consistently among populations: PPLA ranged from 30.2 to 165.7 cm^2, PFSH ranged from 42.6 to 71.2 cm and OrcDen from 8 to 123 ramets/plot.

As for soil physical variables (Table 1, Annex 1), large variations among plots were evident both for stoniness, which had negligible or null value in half the plots but peaked up to 36.8%, and gravel content, ranging from 10.2 to 81.4%. Clay content was very low in all plots (mean value 5.0% ±1.6 SD), and soil texture was for the most part determined by sand (mean value 57.7% ±9.8 SD) and silt (mean value 37.3% ± 9.9 SD).

Nutrient content was generally low ($N\text{-}NO_3^-$ average value was 16.2% ±6.2 SD; PO_4^{3-} average value was 4.4% ±1.5 SD), while organic carbon varied noticeably (from 28.3 to 157.7 g/Kg). Carbonates varied remarkably too (from 40.7 to 292 g/Kg) due to different evolution and weathering processes of the topsoil, yet the pH was quite constant (mean value 7.8 ±0.2 SD).
Aridity Index ranged from 125 for an almost flat East-facing plot to 5250 for a 60° South-West-facing plot.

Principal Component Analysis (Table 2, Annex 1) exhibited a primary axis accounting for 31.4% of the total variance (Eigenvalue=3.45), a second axis accounting for 17.4% of the total variance (Eigenvalue=1.91) and another two axes accounting together for 26.2% of the total variance (Eigenvalues= 1.66 and 1.22). Using the Kaiser criterion (Kaiser, 1960) all four axes could have been retained, but factor loadings for the variables (Table 3 Annex 1) satisfied the correlation threshold of 0.7 only for the first two factors, that were the only ones retained for the selection of variables.

SS, GR, SN, ST factor loadings with respect to factor 1 ranged from 0.74 to 0.83 (absolute value) and AI factor loading with respect to factor two was 0.81 (absolute value), therefore these variables were selected as the only meaningful soil variables to predict *H. adriaticum* fitness (Fig. 2).
Since only SS, SN and AI were uncorrelated (Spearman R< 0.33, p>0.20, Table 4 Annex 1) these three variables were used in the GLZ model building as predictors.

The Aridity Index proved to be a significant predictor for all the three fitness covariates, with a negative effect on PPLA (β=-0.0002; p_W<0.0001) and PFSH (β=-0.0005; p_W=0.009) and positive on OrcDen (β=0.0002; p_W<0.0001) (Table 2).
Stoniness and sand content were significant predictors only for OrcDen, both having a negative effect (SS: β=-0.0529; p_W<0.0001; SN: β=-0.0179; p_W<0.0001) (Table 2).
In general, the relative magnitude of effects was much more skewed to the topography rather than soil properties, since the Wald statistic value was up to three order of magnitude greater for AI than ST or SN (Table 2).

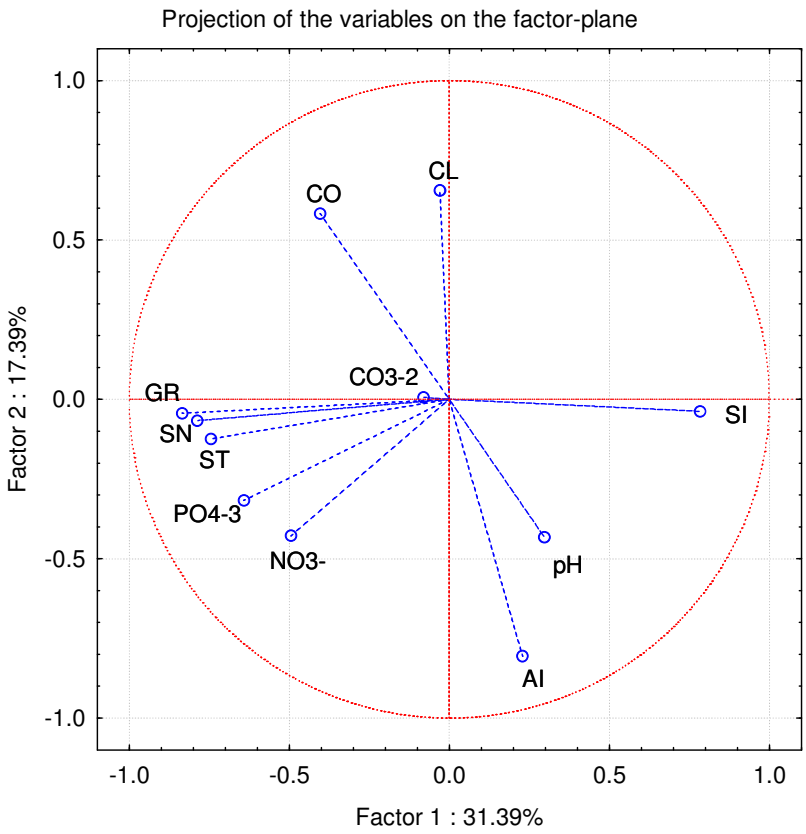

Fig.2: Projection of the soil variables on the plane of the two principal components

Table 2: Results for GLZ models on: A. mean plant leaf area (PPLA); B. mean height of flowering stalks (PFSH); C. ramet density (OrcDen). β=regression coefficients estimates, SE=standard errors of β, Wald and p_W =Wald statistics and p-values for the significance of β, Log-lik=log-likelihood for the model that includes the effects of the given variables and all others before it, $\chi 2$ and p_{LRT} = incremental $\chi 2$ statistic and relative p-value.

A. Mean plant leaf area (PPLA, Poisson distribution, Log Link Function)

	β	SE	Wald	p_W	Log-lik	$\chi 2$	p_{LRT}
Intercept	4.7264	0.11120	1806.420	0.000	-154.4		
Stoniness (%)	0.0007	0.00245	0.098	0.754	-153.8	1.239	0.266
Sand (%)	0.0017	0.00279	0.391	0.532	-153.2	1.199	0.273
Aridity Index	-0.0002	0.00002	113.461	<0.0001	-84.4	137.659	<0.0001

B. Mean height of flowering stalks (PFSH, Normal distribution, Log Link Function)

	β	SE	Wald	p_W	Log-lik	$\chi 2$	p_{LRT}
Intercept	3.9775	0.13426	877.6	<0.0001	-28.1		
Stoniness (%)	0.0039	0.00313	1.6	0.206	-27.4	1.473	0.225
Sand (%)	0.0033	0.00326	1.0	0.312	-27.1	0.497	0.481
Aridity Index	-0.0005	0.00002	6.8	0.009	-23.6	7.091	0.008

C. Ramet density (OrcDen, Poisson distribution, Log Link Function)

	β	SE	Wald	p_W	Log-lik	$\chi 2$	p_{LRT}
Intercept	4.1159	0.16415	628.7	<0.0001	-300.7		
Stoniness (%)	-0.0529	0.00638	68.5	<0.0001	-264.8	71.788	<0.0001
Sand (%)	-0.0179	0.00397	20.3	<0.0001	-253.9	21.724	<0.0001
Aridity Index	0.0002	0.00002	105.1	<0.0001	-205.9	96.092	<0.0001

4.4. Discussion

Our study revealed that physical soil properties are more relevant with respect to chemical properties in predicting the fitness of *H. adriaticum*.

Sampled soils were characterized by a considerable gravel content, since they were thin, scarcely-evolved, occurring on slopes with a significant rate of erosion (Entisols and Inceptisols according to USDA Soil Taxonomy, Soil Survey Staff, 1999). Sand content exceeded 50% for three-quarters of samples, with a negligible percentage of clay (5.0% on average), thus, according to USDA Soil Survey Manual (Soil Survey Staff, 1993), soil can be classified into "loam" and "sandy loam" textural classes. Given such physical attributes, the estimated water retention capacity was low and edaphic aridity was pronounced (Giordano, 1999).

This could explain the results of the GLZ model on *H. adriaticum* ramet density (OrcDen) where stoniness and sand percentage were both significant negative predictors. In fact, as drainage and water shortage increase, the likelihood of seed germination and/or establishment decreases. Such a process can be classified as a resource limitation-based habitat filter, where the key resource is water. This pattern was reported for other terrestrial orchid species (e.g. *Gymnadenia conopsea*, Scott and Carey (2002); *Goodyera pubescens*, Diez (2007); *Neottia ovata*, Jacquemyn *et al*. (2015)). Moreover, soil moisture is known to act indirectly on orchid recruitment, since it impacts the availability and biomass of fungal symbionts involved in seed germination and nourishment of heterotrophic seedlings (Osono *et al*., 2003; Izzo *et al*., 2005).

However, water seems to be a limiting resource only at the recruitment stage of *H. adriaticum*, since stoniness and sand percentage did not affect plant leaf area and flowering stalk height. In other words, once the selection based on water limitation has operated at the seed or seedling level, it no longer affects well established plants. This might be due to the fact that water shortage is particularly significant on the ground surface (in most cases covered by stones and gravel) but became less severe a few centimetres deeper, were orchid roots and tubers are buried in finer soil aggregates and water stress is reduced with respect to the surface (Wine et al., 2015).

However, the "topographical" Aridity Index (AI) proved to be a significant positive predictor of ramet density which is favoured by relatively harsher topographical conditions, perhaps because here the most favourable habitat features for plant establishment were found. In fact locations of steeper slopes and most sunny aspects were also characterized by a greater portion of bare ground or eroded soil where seeds can lodge and seedlings have enough space for growth. Nevertheless, AI had a negative impact on plant leaf area and flowering stalk height, probably because the same morphological features that favour orchid establishment in the early phases of

their life cycle impose non-optimal conditions (in term of drought stress and excessive heat) for vegetative growth.

Therefore it can be argued that soil physical properties and local morphology have counteracting effects on the regeneration niche of *H. adriaticum*, in the sense that abiotic harshness imposed by the former is detrimental due to the difficult-to-meet ecological requirements for seed germination and seedling survival on the soil surface, while the latter is beneficial from a microsite limitation perspective since it is correlated to site availability for seed lodging and seedling growth (Eriksson and Ehrlen, 1992; Clark *et al*., 2007).

Once juvenile plants have established, the main driver of vegetative fitness is represented by local morphological features, summarized in the Aridity Index. This does not necessarily mean that soil physical properties become irrelevant, rather that the relative magnitude of effects is much more dependent on the topography.

This study was aimed at evaluating the effects of local soil properties and morphology on the fitness of *H. adriaticum* populations. Despite some studies having found significant relationships between soil chemical factors and orchid fitness (e.g. Silvertown *et al*., 1994; McKendrick, 1996; Batty *et al*., 2001; Bowles *et al*., 2005), the present studyfound that physical properties are the most important driver of *H. adriaticum* leaf area, plant height and population density. Results were interpreted from the perspective of microsite availability and resource limitation for seed germination and seedling establishment, since the tested physical variables can be interpreted as a proxy of soil water retention, runoff, and microclimatic harshness.

5. Assessment of germination capacity of seed from contrasting orchid populations and germination promotion via lignin modifying enzymes (LME)

5.1. Assessment of orchid population germination capacity

5.1.1. Introduction

Terrestrial orchids are characterized by a combination of life history and reproductive traits that greatly limit their chance of expansion, even in a favourable habitat. In fact these species have a short life span allowing rare flowering events followed by several dormant phases (Shefferson, 2002; Gregg, 2004), during their subterranean phase mortality may be high due to predation or damage of underground tubers or rhizomes (Kretzschmar *et al*., 2007; Jacquemyn *et al*., 2009), vegetative propagation is limited in space and time (Wells and Willems, 1991) and flower visits by pollinators may be scarce leading to low levels of fertilization (Walsh *et al*., 2014). Therefore copious recruitment from seeds is fundamental to ensure the persistence of orchid populations over time (Hutchings, 2010).

Despite the record numbers of seeds produced per fruit (up to $4*10^6$; Arditti, 1981), several factors limit the probability of an orchid seed becoming an established plant. Firstly, seed dispersal is limited to a distance of few meters from the mother plant but only a small portion of seeds will arrive in a suitable germination environment providing adequate gaps for plant growth, resource availability and microclimate, also known as microsite limitation (Clark, 1999). A set of biotic factors, such as intraspecific competition, predation and pathogen attacks may affect the survived plants in the rest of their lifespan further restricting overall population growth (Jacquemyn, 2007a). Moreover, changes in management regime of habitats can have a dramatic impact on orchid populations, both in terms of size and demographic structure (proportion of reproductive or dormant ramets). Hutchings (1987a) reported a sudden overwhelming increase in the size of the biggest UK population of *Ophrys sphegodes* when management regime shifted from heavy-disturbing cattle breeding to more meadow-respectful sheep grazing.

The same author observed that population size, number of flowering plants and number of recruits of *Orchis militaris* increased promptly after the removal of the tree canopy above the species location (Hutchings et al., 1998) while *Cephalanthera longifolia* and *Gymnadenia conopsea* spontaneously flowered at Monte Barro Regional Park (LC, Italy) the year after the removal of invasive weed species in the formerly shaded habitat where they appeared to have disappeared for years (Pierce and Belotti, 2011).

Habitat features may also impact directly the quality of seeds, since the parental environment is known to affect seed maturation and dormancy characteristics within the same species. High or low temperatures, vegetation-limited light intensity, mineral nutrients availability and drought affecting mother plants are likely to modify seed size, weight (Baker, 1972; Lee and Fenner, 1989; Eriksson, 2000) and morphology (Lacey, 1997), while differences in temperature, light quality (wavelength), soil moisture and time of fruit ripening determine a difference in the degree of dormancy (Gutterman, 2000; Baskin and Baskin, 2014).

Eventually, a gene flow-limiting landscape structure and isolation of populations by distance usually have a highly detrimental effect on the genetic variability of populations. The smaller and more isolated these are, the more likely it is that they will suffer from genetic erosion, and genetic divergence among populations increases due to the effects of genetic drift and inbreeding (Young, 1996). The phenomenon is particularly pronounced for small-density populations and for herbaceous plant species (Vekemans and Hardy, 2004), and is likely to produce adverse effects on the number of viable seeds (Spigler and Chang, 2009), seed size/mass (Stephenson *et al*., 2004) and germination percentages or rates (Ferdy *et al*., 2001; Juillet *et al*., 2007).

The consequences of these detrimental processes can lead to a progressive reduction in the reproductive capability of a population, undermining its survival and chances of recovery in the future (Jacquemyn *et al*., 2007b).

The aim of this Section was to investigate the germination capacity (i.e. the total germination) of terrestrial orchid populations growing in similar habitats (dry grasslands and nearby xerophilous scrublands or hedges) but subjected to different management regime (completely abandoned to yearly mowed) and vegetation structure (only herbaceous or surrounded by a woody species canopy).

Two hypothesis were tested: a) different populations of orchid species do not have the same germination capacity; b) differences in germination capacity can be explained by differences in population demographic parameters and/or according to habitat type.

5.1.2. Material and methods

Target species and seed collection

The hypothesis were tested for 5 populations of *Ophrys sphegodes*, 5 populations of *Anacamptis morio* and 10 populations of *Himantoglossum adriaticum* distributed among the Lessini-Berici-Euganean Hills in the Veneto Region, NE Italy (Fig. 1). These populations were ideally enclosed in a permanent 2 × 2m sampling quadrat and georeferenced in Spring 2013 at the peak of their respective flowering times (*O. sphegodes*: 1st week of April; *A. morio*: 3rd week of

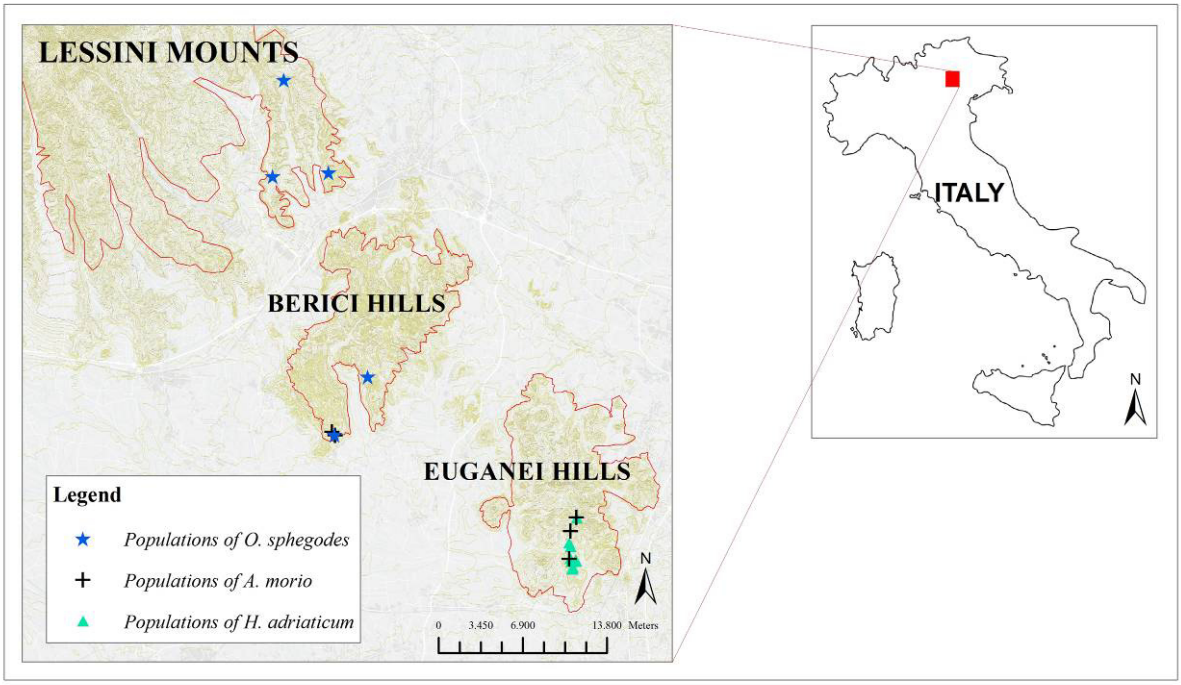

Figure 1: Distribution of the tested populations

April; *H. adriaticum*: 3rd week of May). Demographic censuses were performed recording the density of all orchid ramets (an estimation of the population size per unit area) and flowering ramets per plot ($4m^2$).

For each plot, 4 to 10 ramets were tagged and monitored until the end of June 2013 to determine the mean proportion of flowers that were fertilized and developed into fruits (FFR, see Chapter 2). The ripening of the fruits were monitored weekly after the flower fertilization and capsules were collected at the time when longitudinal fissures appeared after a gentle squeezing, to ensure both a complete maturation of the seeds and the lack of parasitosis which are likely to quickly develop after the fruits opening (Pierce and Belotti, 2011).

Fresh-collected capsules were temporarily stored in paper envelopes and completely opened in the lab where seed batches where cleaned under a stereoscope using forceps. Pure seed batches were than stored in airtight glass vials at 15°C and 15% RH following the international standards (FAO/IPGRI, 1994).

In-vitro experiment for hypothesis testing

In October 2014 seed of each population of each species was sown *in-vitro* to measure the total germination capacity. Seed was surface sterilized by soaking in 1.5 ml of 0.4% NaOCl bleach solution (equivalent to 0.25% active chlorine) containing 0.1% of liquid detergent (SACI Industrie Spa, Perugia, Italy) as surfactant agent for 20 minutes. Only for *H. adriaticum* a solution of 0.8% NaOCl (equivalent to 0.5% active chlorine) was used for 15 minutes in place of the former to

weaken the hard structure of the seed testa, which is likely to hinder germination due to low water permeability (Rasmussen, 1995). After surface sterilization, seed was rinsed 5 times with sterilized distilled water and sown on agar medium in 6 cm-diameter ventilated Petri dishes. The entire process was carried out under a laminar flow hood (Mod. 1200 FLO, Permax Srl, Milano, Italy) with sterilized lab utensils.

The agar sowing medium used was based on Malmgren's orchid medium (Malmgren, 1996), modified with the addition of 0.5 g L^{-1} Peptone and 0.1 mM 6-benzylaminopurine (BAP), since these compounds have proven to be effective in aiding germination of several terrestrial orchid species (Pierce and Belotti, 2011). Moreover, 1 U/Petri dish of Laccase was incorporated in the agar medium using cool sterilization, since this enzyme has also proven to be effective in enhancing orchid seed germination (see detail in section. 5.3 of this work). The pH of the agar medium was fixed at 6.5 for all the target species using 0.1 N NaOH or HCl prior to sterilization of the medium in the autoclave (Mod. 760, Permax Srl, Milano, Italy) at a temperature of 121 ºC and a pressure of 1 MPa.

According to the amount of seed available, 12 to 22 replicates per population were prepared (after a week, a small amount of contaminated Petri dishes (<2 units/population) were removed and were not considered further for the analysis). All Petri dishes were sealed with laboratory film (Parafilm) and wrapped in two layers of aluminium foil to prevent potentially detrimental exposure to light (Nikabadi *et al*., 2014) and then placed in a growth chamber (Snijders Economic Deluxe, Thermo-Lab, Codogno, Italy) at 20/10°C day/night temperature. Petri dishes were checked monthly for evidence of germination and final germination percentage was determined at the stereoscope six months after sowing when the number of germinated seeds demonstrated no further increase. A seed was considered germinated when it entered the developing phase II described by Butcher and Marlow (1989), i.e. the embryo swelled enough to split the seed coat and form a white protocorm. The proportion of seeds germinated, calculated as the ratio between the number of seeds in phase II and the number of seed tested in each Petri dish, was considered the germination capacity of a seed batch in a single replicate.

For *H. adriaticum* populations, characterized by a very low germination capacity (<4%), the proportion of ungerminated seeds with embryo or lacking the embryo (unfertile seeds) were also determined to discriminate between those seeds that failed to germinate due to developmental problems or dormancy and due those seeds simply lacking fertilization.

Data Analysis

To compare the germination capacity between populations (i.e. the mean proportion of seeds germinated among all replicates referring to each population), a Kruskall-Wallis analysis of variance (ANOVA) was performed in STATISTICA 8.0 (StatSoft Inc, 2007) followed by a post-hoc multiple comparison of mean ranks for all groups (Siegel and Castellan, 1988). The nonparametric ANOVA was chosen because both original and arcsin-square root-transformed data failed to match the requirements for the parametric tests.

Moreover, a Kolmogorow-Smirnov test was performed on two groups of *H. adriaticum* populations to test for differences between two habitat types (closed *vs*. open vegetation structure) in germination capacity, germination capacity of fertile seeds only (i.e. the proportion of seeds with embryos that entered the phase II) and the proportion of unfertile seeds (=number of seeds lacking the embryos/number of seeds tested). Populations were classified as belonging to closed (C) vegetation structure if a tree layer was present in their respective plots (vegetation data of Chapter 2 were used), otherwise they were classified as belonging to "open" (A) vegetation structure.

5.1.3. Results

Anacamptis morio

Populations of *Anacamptis morio* (Table 1) coming from managed meadows showed larger population sizes and a higher proportion of flowering ramets than populations found in unmanaged areas. In particular, the largest population (440 individuals) was found in a yearly moved grassland, where the landowner usually practices haymaking in mid-June, when *A. morio* have already dispersed seed. A "partly" managed grassland (34) means that the landowner does not mow the grassland regularly, but mowing is practiced at least once every three years to avoid the establishment of woody species.

Populations coming from unmanaged areas were smaller and occupied vegetation gaps within scrublands and woodland, but retained a high proportion of fertilized flowers (FFR).
Significant differences in germination capacity were evident between populations 34 (18.4%, $p \leq 0.001$), 26 (59.4%, $p \leq 0.05$) on the one hand and populations 30, 31, 44 (78.6-85.3%) on the other (Table 1 Annex 2). Therefore, the main result was that germination capacity is population-specific (Fig. 2), however these populations belonged to both managed and unmanaged habitats and there was not a clear separation between more and less performing populations according habitat type or population size or FFR.

Table 1: Characteristics of tested *Anacamptis morio* populations. FFR denotes the proportion of fertilized flowers, on a scale of 0 to 1.

Population (Plot number)	Proportion of seeds germinated (%)	S.D.	Population size (number of ramets)	Number of flowering ramets	FFR	Habitat
26	59.4	7.84	35	33	0.47	Open unmanaged meadow
30	85.3	8.57	33	18	0.56	Woodland/meadow mosaic
31	78.6	6.96	113	24	0.25	Scrubland (abandoned vineyard)
34	18.4	7.99	115	40	0.19	Open partly managed meadow
44	81.7	7.41	440	320	0.45	Open managed meadow

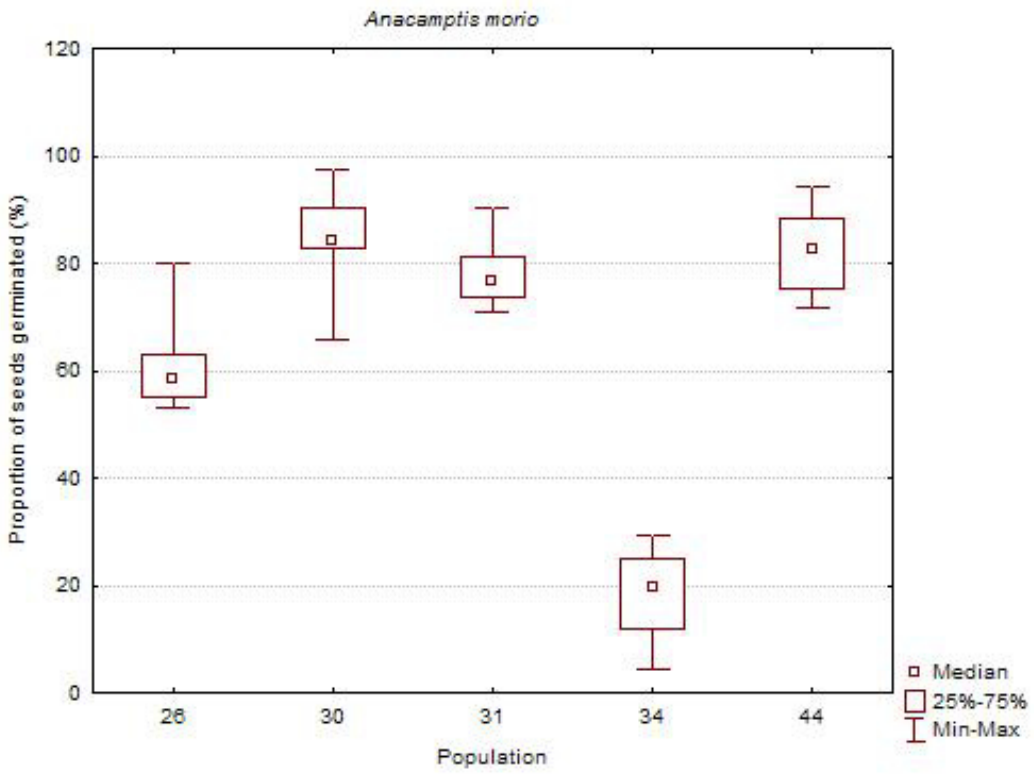

Figure 2: boxplots representing the total germination of different *A. morio* populations.

Ophrys sphegodes

Populations of *Ophrys sphegodes* (Table 2) from managed meadows exhibited larger size and a greater number of flowering ramets than populations found in unmanaged areas. The largest (393 individuals) was found in a top-hill south-facing grassland where the local agency for forestry and land management (Veneto Agricoltura) continuously maintains the woodland edges preventing invasion of woody species into the grassland.

Populations coming from unmanaged areas were smaller and made by few flowering individuals. They occupied marginal areas often invaded by woody species and/or facing forms of degradation, but they retained a germination capacity comparable or even larger than populations of managed areas. In particular, population 21 was placed in a remnant dry-grassland jeopardized by

motocross trails where rain runoff causes strong erosion. This population was composed of only 7 flowering ramets but was among the best performing populations (70.4% final germination).

Although germination capacity is a population-specific characteristic of *O. sphegodes* (Fig. 3), no separation according habitat types or population demographic features was detectable.

In fact, extremely large-sized population 23, which benefited from favourable management practices, had almost half germination capacity (36.9% *vs*. >60.9%, p<0.001) of the best performing but smaller populations 13, 21, 29 (Table 2 Annex 2), found in meadows mowed just once every three years or not at all.

Table 2: Characteristics of tested *O. sphegodes* populations. FFR denotes the proportion of fertilized flowers, on a scale of 0 to 1.

Population (Plot number)	Proportion of seed germinated (%)	S.D.	Population size (number of ramets)	Number of flowering ramets	FFR	Habitat
7	48.6	10.02	46	13	0.23	Woodland/meadow mosaic
13	68.3	8.06	98	70	0.18	Partly managed meadow
21	70.4	6.52	10	7	0.09	Eroded scrubland
23	36.9	5.82	393	169	0.05	Managed meadow
29	60.9	6.99	173	62	0.38	Partly managed meadow/hedge mosaic

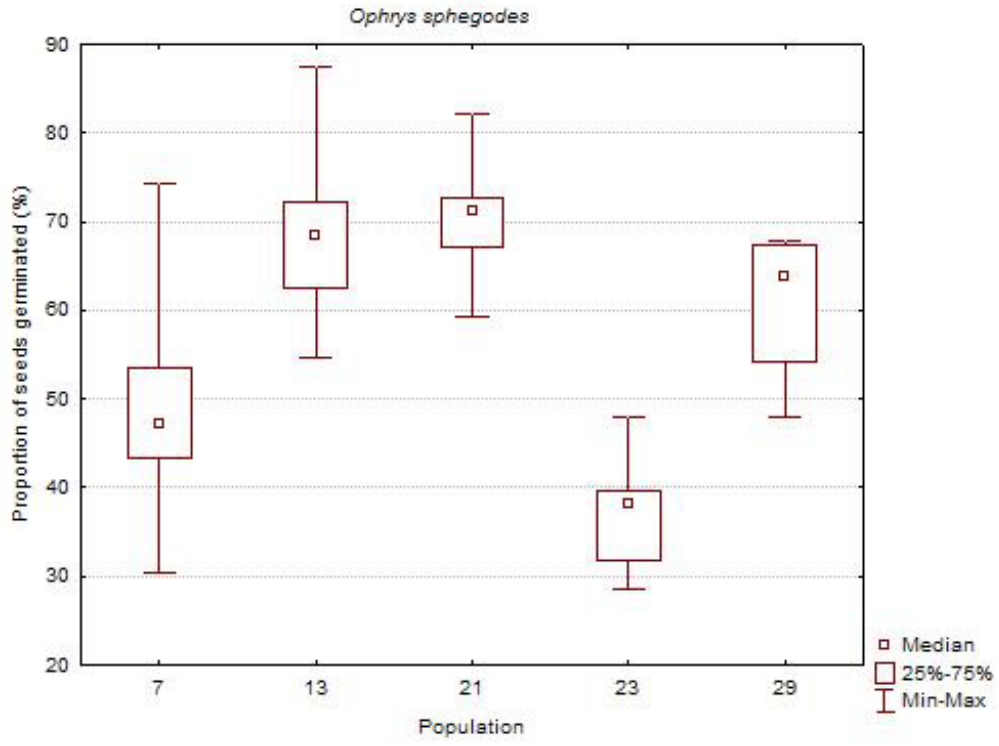

Figure 3: boxpolots representing the total germination of *O. sphegodes* populations.

Himantoglossum adriaticum

Populations of *H. adriaticum* (Table 3) came from a wider variety of habitats than *A. morio* and *O. sphegodes*. The latter were placed in managed or unmanaged meadows, in wide grasslands or small remnants between woody vegetation, but never in the woody vegetation understory. On the contrary, *H. adriaticum* populations were found in open extensive meadows (>2 Ha), narrow (5-10 meters-wide) strips of meadow enclosed between hedges, ecotonal areas between woodland and meadow, inside-hedge understory, both in managed and unmanaged regimes.

Despite their provenance, all populations showed a very low germination capacity both in terms of the proportion of seeds germinated (<3%, Fig.4) or the proportion of embryos (≤5%). Moreover, the proportion of unfertile seed was very high for the greater part of them (four populations had more than 40% of seeds with a reduced or absent embryo).

The largest populations came from managed meadows (36, 51), had the largest number of flowering ramets and a high proportion of unfertile seeds. Smallest populations came from ecotonal areas between meadows and woody vegetation (45, 57) or unmanaged areas with shrubs/trees (41, 47, 50). Between-population tests for differences (Table 3 Annex 2), showed that only populations 41, 50, 59 had a significantly ($p<0.05$) lower total germination (<1.1%) with respect to populations 36 and 45 (1.9 to 2.8%) but no population features were associated with such a result (Table 3).

Table 3: Characteristics of tested *H. adriaticum* populations. A=open vegetation structure, C=closed vegetation structure. FFR denotes the proportion of fertilized flowers, on a scale of 0 to 1.

Population (Plot number)	Habitat	Population size (number of ramets)	Number of flowering ramets	Proportion of seeds germinated (%)	Proportion of embryos germinated (%)	Proportion of unfertile seeds (%)	FFR
36	Managed open meadow (A)	115	52	1.9	3.5	42.4	0.41
41	Unmanaged eroded shrubland (C)	8	4	0.5	1.1	42.5	0.21
45	Managed hedge/meadow ecotone (A)	15	10	2.8	5.0	44.1	0.20
46	Unmanaged woodland/ grassland mosaic (A)	62	24	1.5	1.8	11.1	0.01
47	Abandoned Vineyard (A)	13	5	2.3	4.4	43.7	0.20
50	Abandoned hedge (C)	15	11	0.8	1.0	23.8	0.01
51	Managed closed meadow (A)	123	31	1.8	3.0	37.0	0.10
57	Managed woodland/ meadow ecotone (C)	9	7	1.1	1.3	15.7	0.22
58	Unmanaged hedge/ meadow ecotone (C)	37	13	1.2	1.5	21.4	0.34
59	Unmanaged road side/woodland (C)	28	21	1.1	1.5	28.0	0.04

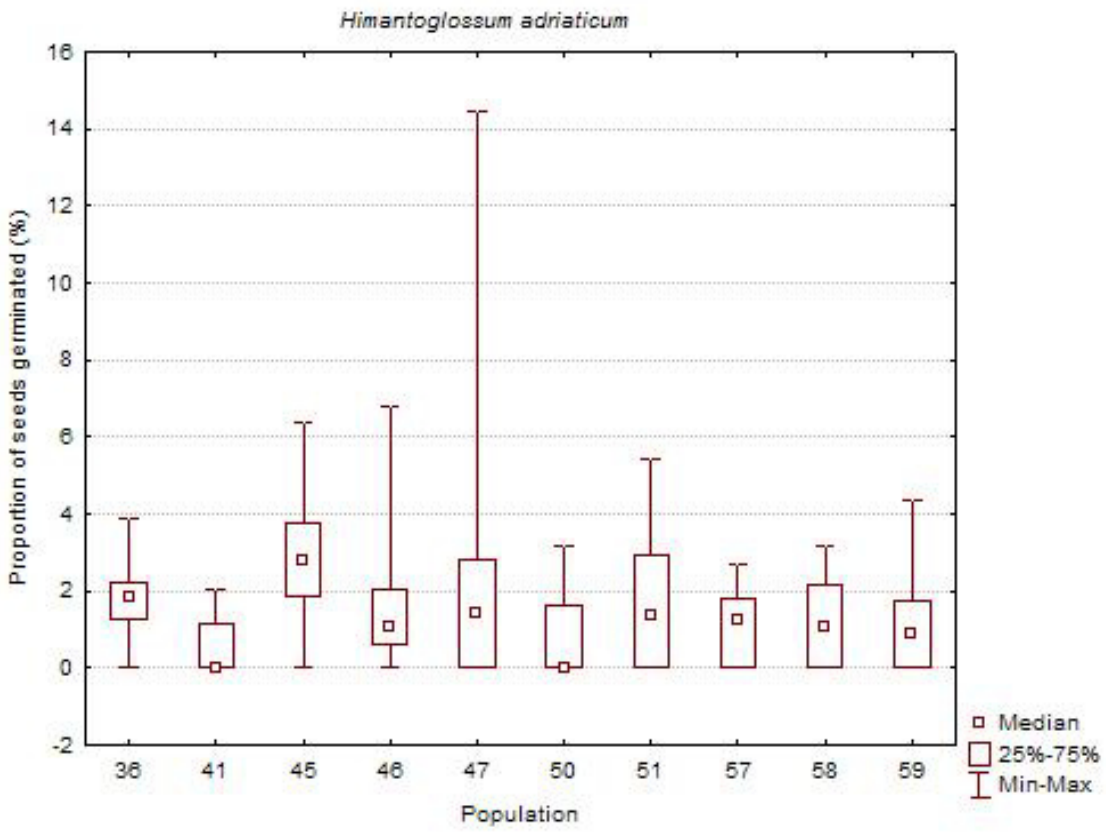

Figure 4: boxpolots representing the total germination of *H. adriaticum* populations.

On the contrary, the habitat type was associated with populations differences: populations 36 and 45 belonged to managed areas with no woody vegetation inside the plot, populations 41, 50, 59 belonged to unmanaged areas and are literally "hidden" in the woody vegetation understory.

Grouping all the populations according the open ("A") or closed ("C") vegetation structure of their respective locations revealed a clear difference in all the three variables considered in the germination test (Fig. 5). The Kolmogorov-Smirnov test (Table 4 Annex 2) proved a significant separation between "A" and "C" populations in the proportion of seeds germinated (1.9% *vs.* 1.1%, $p<0.05$, Fig. 6), the proportion of embryos germinated (3.4% *vs.* 1.4%, $p<0.001$, Fig. 7) and the proportion of unfertile seeds (42% *vs.* 20%, $p<0.001$, Fig. 8).

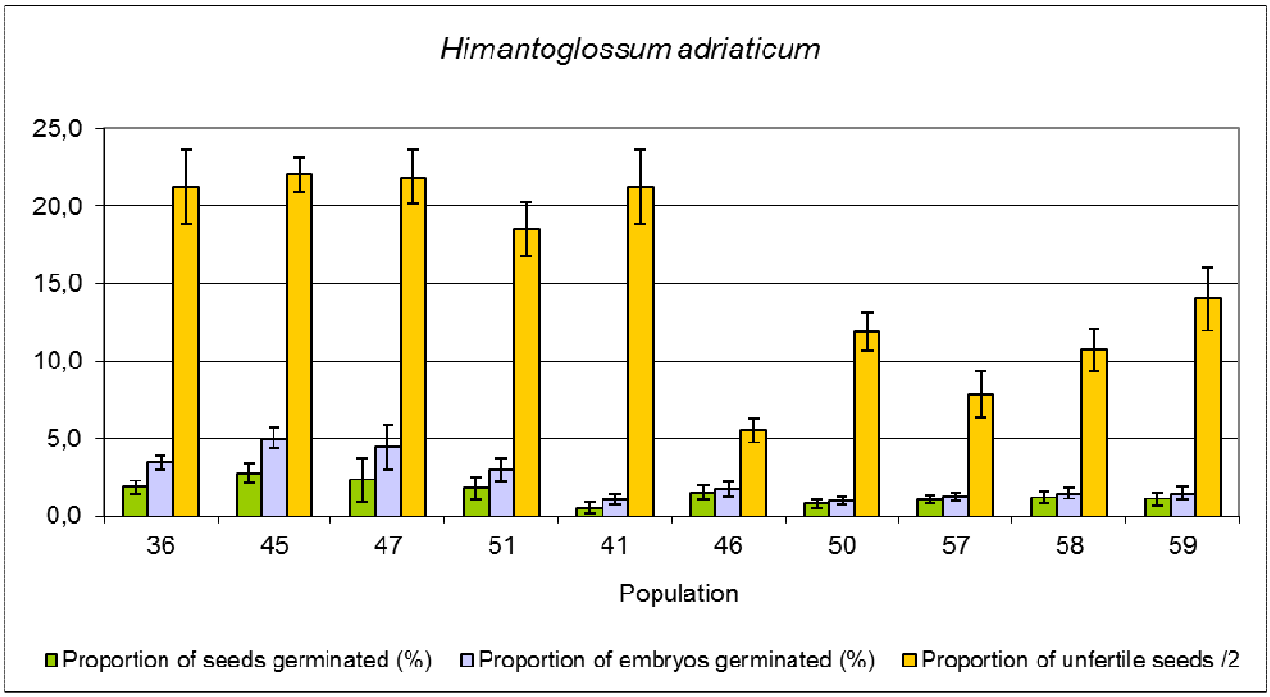

Figure 5: results of germination test of *H. adriaticum* populations referring to "open" (5 populations on the left side) and "closed" (5 populations on the right side) vegetation structure. Bars represent standard errors of means.

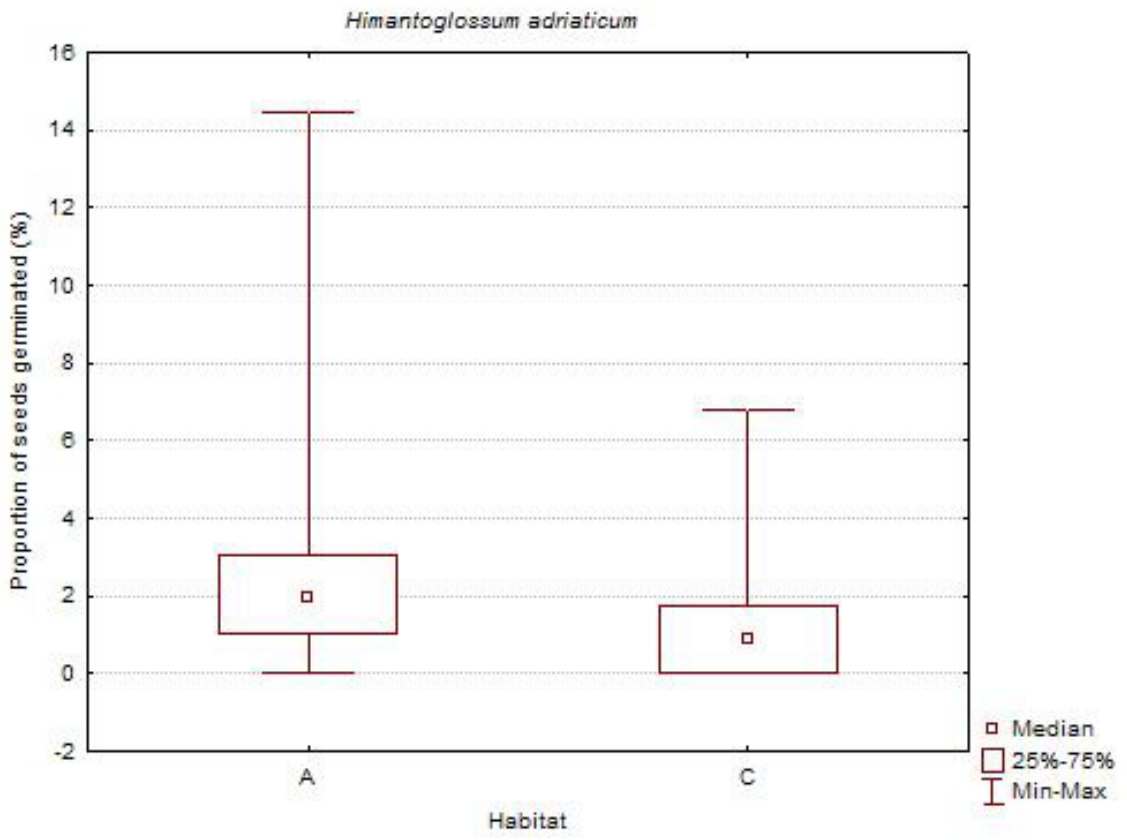

Figure 6: boxplots representing the total germination of *H. adriaticum* populations referring to open ("A") and closed ("C") vegetation structure.

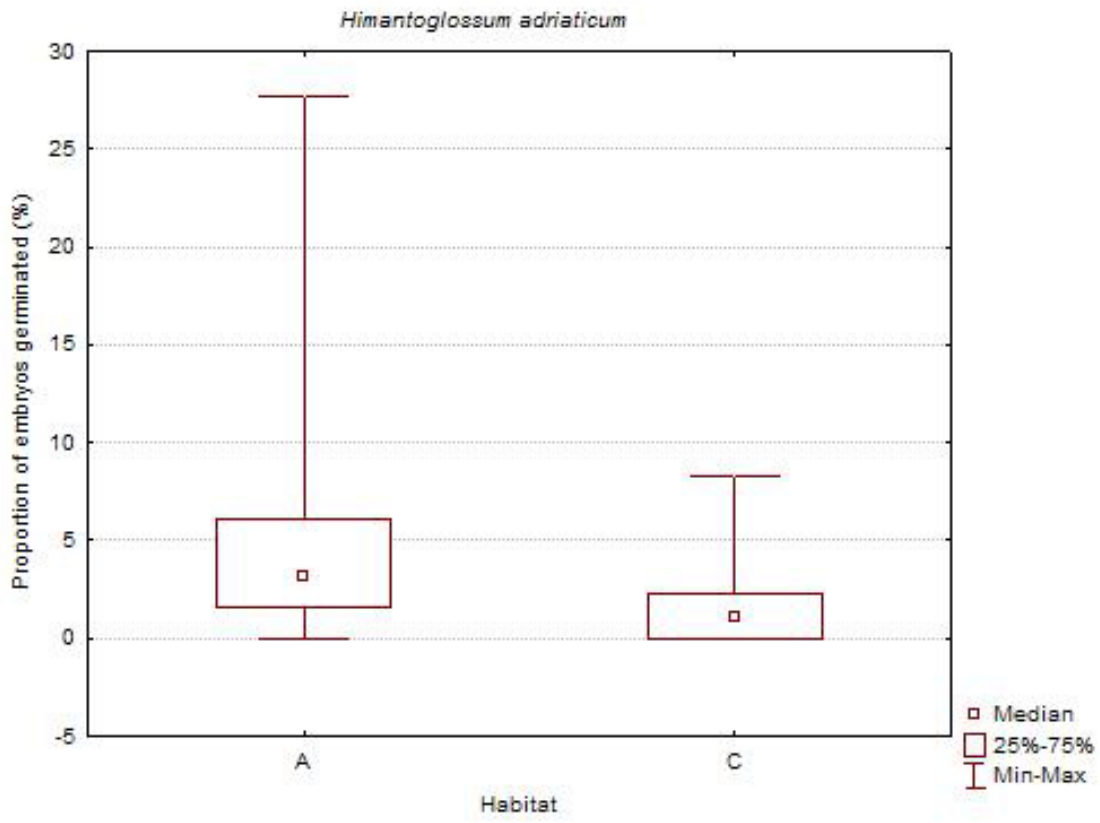

Figure 7: boxplots representing the total germination of embryos of *H. adriaticum* populations referring to open ("A") and closed ("C") vegetation structure.

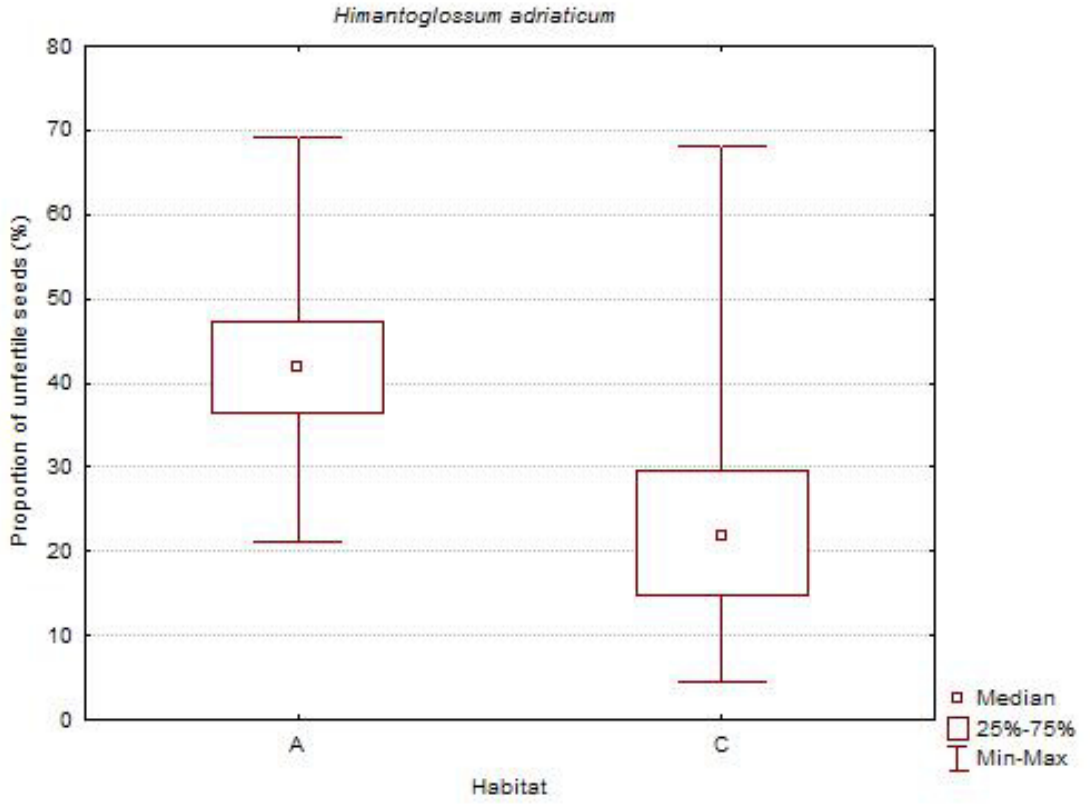

Figure 8: boxplots representing the unfertile seeds proportion of *H. adriaticum* populations referring to open ("A") and closed ("C") vegetation structure.

5.1.4. Discussion

Anacamptis morio and *Ophrys sphegodes* populations have a species-specific germination capacity, but this is not linked to habitat type or population demographic features or visitation rate to flowers (FFR). It is quite surprisingly that for *A. morio* the best performing populations, whose germination capacities (78.6 to 85.5%) were not significantly different, came from contrasting vegetation structures: a regularly mowed grassland and an abandoned scrubland or even unmanaged woodland/meadow mosaic, where grassland vegetation is literally overwhelmed by a tree canopy. Moreover, in this closed structure, where the surrounding woodland isolates the remaining few *A. morio* individuals from nearby populations, the FFR is the highest (0.56). It is expected that small and isolated populations suffer from pollinator limitation (Rathcke, 1983; Donaldson, 2002; Kirchner *et al*., 2005). On the contrary, in this case the high FFR could be explained by isolation since *A. morio* was among the first species to flower (mid-April) in the considered habitat and pollinators appear to have indulged in visiting the few (18) ramets. The reason might be due the fact that the target orchid inflorescences were the only coloured and scented spot in the middle of a mainly woody vegetation and this behaviour appears to have translated into a higher number of fertilized flowers. On the other hand, *A. morio* being self compatible (Nilsson, 1984), an higher frequency of interplant flights may favour geitonogamy (Karron *et al*., 1995), but whether this was the case, it did not affect germination capacity.

The worst outcome in total germination (18.4%) belonged to a population found on a North-facing meadow, mowed once every three years, which was also the least fertilized population (FFR=0,19). Despite a low FFR being recorded also among the best performing populations (0.25), and thus it is not unequivocally related to low germination performance, it could partially explain the low seed quantity and quality in the perspective of resource limitation. In fact the local North-facing shaded slope imposes a solar radiation constraint, which was exacerbated by a rainy sampling season. Pollinator activity is affected by unfavourable weather conditions (Molano-Flores *et al*., 1999; Mustajarvi *et al*., 2001) and whenever it coincides with the short flowering time of orchid species this may result in severe pollinator limitation.

The best-performing populations of *Ophrys sphegodes* came from both seldom-mowed grasslands and eroded scrubland. Among these populations, number 29 was very small (19 flowering ramets) and lived in a small meadow (tens of m^2) completely surrounded by woodland that isolate it from the nearest population (several kilometres away). Nevertheless, it was the most highly visited by pollinators (FFR=0.38) and had the highest absolute value of seed germination capacity (70.4%). Since self-pollination in *O. sphegodes* is possible, although rare (Hutchings, 1998), it can be argued that isolation does not necessarily impose pollinator limitation, confirming

the results found for *A. morio*. It is worth noting that the largest population (169 flowering ramets) had the lowest germination capacity (36.9%) and the lowest visitation rate (0.05), despite its habitat features (0.5 Ha, early- summer yearly-mowed grassland on a south-facing slope) can be considered among the most favourable for the reproductive fitness of this species (Hutchings, 1987b).

Nevertheless, the very high density of flowering ramets could be detrimental to the visitation rate of flowers, since *O. sphegodes* is a sexual deceptive orchid and pollinators tend to "learn" that this species is not rewarding during their vain visits to flowers and thus avoid the highly visible clump of cheater plants (Schiestl, 2005). The consequence is a very low pollen transfer which might translate into low seed quality, although available data are not sufficient to generalise this explanation for all the populations.

Himantoglossum adriaticum exhibited a clear separation between poor and highly performing populations according to the open or closed vegetation structure of the habitat.
Nevertheless, it seems contradictory that higher germination capacity is related to a higher proportion of unfertile seeds and a lower germination capacity corresponds to a larger part of fertile seeds, but the explanation could be in the different level of microclimatic stress and resource availability according to the habitat features. Populations living in "open" habitats experience a major climatic stress (strong solar radiation, heat, drought), but are probably less isolated and benefit from a more significant genetic flux than the populations found in "closed" habitat, which have more available resources (nutrients, water) and are buffered against extreme temperature or drought, but are almost hidden by the woody vegetation and thus experience a greater isolation. It could be argued that *H. adriaticum* uses different strategies according to resource constraints: in "open" habitats there is less seed set because of water shortage and heat, but seed quality is better as populations are less likely to suffer from isolation, while in "closed" habitats *H. adriaticum* has much more water and a favourable micro-environment to invest in growth and tissue development, included fruits and seeds mass, but the quality of its seeds might be low due to gene flow depression or sub-optimal micro-environmental conditions for the maturation of fruits (Pedersen *et al*., 2012).

However, despite the statistically significant differences in germination capacities between the two groups, it must be highlighted that the germination capacity is in any case very low (<4.4%) which could be a sign that inbreeding depression is likely to occur in all the populations.

5.2. Effect of pollen transfer between populations of *Himantoglossum adriaticum*

5.2.1. Introduction

Angiosperms have developed several mechanisms (e.g. self-incompatibility) to avoid self-pollination and reproduce by outcrossing (Liu, 2013), since a larger genetic pool within a breeding population offers genetic advantages to the offspring with respect to inbreeding (Holsinger, 1991).
On the other hand, several species use self-compatibility as a reproductive assurance in case of mates and/or pollinator vector scarcity, but the selfed offspring are usually less viable than outcrossed ones (Herlihy and Eckert, 2002).

Small-sized and isolated populations are likely to suffer from inbreeding depression, that is the accumulation of detrimental effects on their fitness driven by the repeated breeding with closely-related individuals (Kelly, 2005). Two possible explanations have been suggested: a. the reduction in heterozygosity of the inbred offspring; b. the accumulation of rare and deleterious mutations, protected from natural selection by at least partial recessivity (Crow, 1993).
Inbreeding depression is reported to have important implications for plant population ecology and conservation, since it depresses the seed set, germination, survival and resistance to stress (Keller and Waller, 2002; Shiau et al., 2002), while the cross-breeding enhancement of offspring fitness-relate traits as fruit set, fruit size and mass is fundamental to species reproduction and of high value in agriculture (Spinardi and Bassi, 2012; Azevedo *et al*., 2013; Muller *et al*., 2013).

H. adriaticum populations in the Veneto Region are small sized and extremely isolated. There is no evidence for self-pollination in the genus *Himatoglossum* though it is physically possible (Carey and Farrell, 2002) and geitonogamy is reported likely to occur when pollinators indulge in the same inflorescence, even in strongly but not completely self-incompatible orchids (Singer and Koehler, 2003).

Given the very poor germination outcome of *H. adriaticum* populations in Section 5.1, the aim of the following study was to test the possibility of inbreeding depression using inter-population artificial cross-pollination and its effectiveness for enhancement of germination.
The hypothesis tested was that ramets of *H. adriaticum* from isolated and smaller-sized populations fertilized with pollen coming from larger, non-isolated populations exhibit a greater germination capacity than when cross pollinated within the same population.

5.2.2. Material and methods

Target populations

Three small and isolated populations of *H. adriaticum* (Fig.9) were selected in the Pre-alpine hills to the West of the city of Vicenza, where dry grasslands are extremely reduced in surface (< 0.2 Ha) and completely surrounded by woodlands, areas under cultivation (particularly vineyards) and villages. Two of these populations, labelled URB-W and URB-H, are found at a distance of just 0.5 Km from one another but are separated by hedges, olive groves and maize fields near the village of San Urbano, the former population in the ecotonal area between a xerophilous wood and a dry meadow, the latter in a top-hill dry meadow closed on one side by a hedge. A third population, labelled CREA, was found along a roadside, completely overshadowed by a tree canopy, near the village of Creazzo. The area is sparsely inhabited and no regular maintenance of roadside vegetation takes place.

One large population of *H. adriaticum*, named "GAMB" was selected in the district of the Berici Hills, on the south-facing slope of Mount Gamborello, village of San Germano, with several hundred ramets spread in yearly-mowed portions of a terraced dry meadow partly separated each other by discontinuous hedges.

A small chance of naturally occurring cross pollination is possible only between URB-W and URB-H, the only populations separated by an agro-forest matrix in a short distance, since CREA and GAMB are separated from each other and from the former populations by tens of square kilometres of urban-industrial areas over a long distance (4.5 to 18 Km).

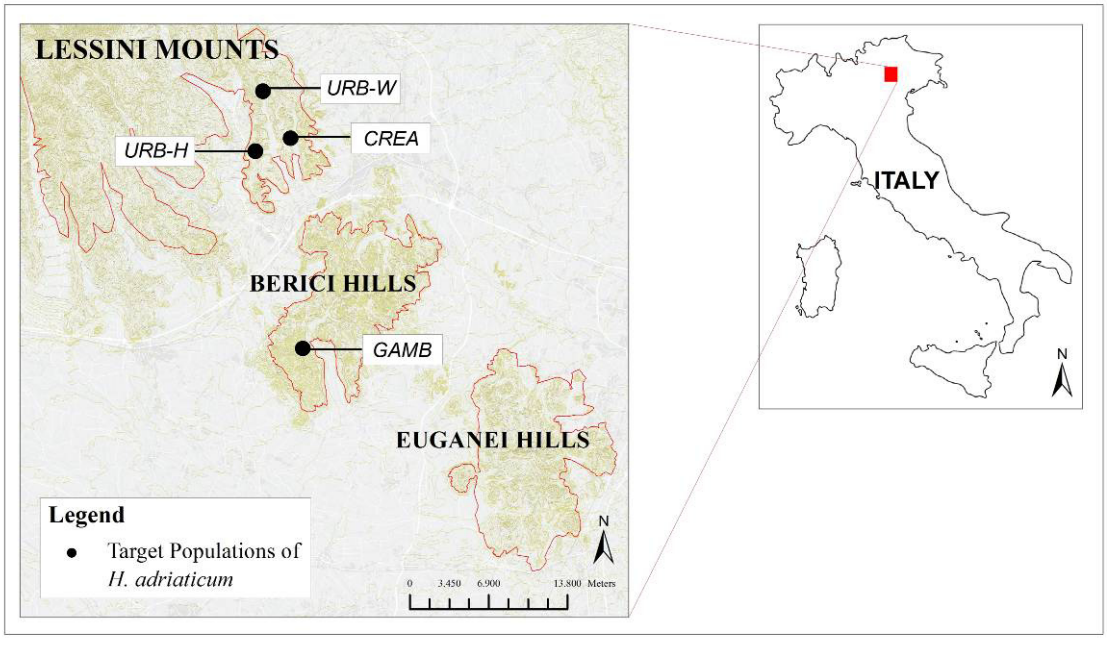

Figure 9: location of *H. adriaticum* populations involved in the cross-pollination test

Pollen transfer and hand pollination

URB-W and CREA were chosen as the small-sized populations to test for the effectiveness of the large-population (GAMB) pollen transfer for increasing total germination. URB-W was also the pollen donor population for URB-H, tested for the effectiveness of pollen transfer between two small but less isolated populations (Table 4).

Pollinia were collected from 10 to 50 (depending upon the population size) healthy *H. adriaticum* ramets of the donor population at the peak of the flowering season in 2014 (i.e. the third week of May) using toothpicks. To prevent desiccation before use, pollinia were placed in a 9 cm-diameter Petri dish, sealed in the field with paper adhesive tape and kept in a coolbag (15 ± 2°C) until returning to the lab facilities where they were stored in the fridge (4°C). The day after collection, pollinia were used to pollinate the target population's seed donor ramets.

Three to five healty ramets (Table 4) were selected and marked with a transparent plastic label. All pollinia belonging to them were removed to avoid accidental autogamy and used in within-population cross pollination of URB-W, URB-H, GAMB (control treatments). Few flowers already pollinated by insects were damaged and only non-pollinated flowers deprived of their original pollinia were left on the tagged ramets. Only completely intact and ripened pollinia (dark grey in colour) of the donor populations were used to pollinate the flowers of the recipient population.

All the tagged ramets were monitored weekly after pollination to check for fruit development and maturation, and ripened capsules were harvested in the last week of June 2014. All fruits referring to the same tagged ramet were placed in the same paper envelope at the time of harvesting, cleaned in the lab by hand, and stored as independent seed in airtight vials following the FAO international standard (FAO/IPGRI 1994).

Table 4: *H. adriaticum* populations involved in between-population cross pollination experiments and the experimental design of crosses

CROSS code	Seed donor habitat	Pollen donor habitat
CR1	URB-W: woodland/meadow ecotone	GAMB: hedge-enclosed meadows
	number of flowering ramets: 27	number of lowering ramets: >500
	seed donors ramets: 3	pollen donors ramets: 50
	distance between populations: 18 Km	
CR2	CREA: unmanaged road side/woodland	GAMB: hedge-closed meadows
	number of lowering ramets: 28	number of lowering ramets: >500
	seed donors ramets: 5	pollen donors ramets: 50
	distance between populations: 16.5 Km	
CR3	URB-H: hedge-sided meadow	URB-W: woodland/meadow ecotone
	number of lowering ramets: 35	number of lowering ramets: 27
	seed donors ramets: 5	pollen donors ramets: 10
	distance between populations: 0.5 Km	

In-vitro experiment for hypothesis testing

Seeds were sown in a sterile environment using the Malgrem's modified medium described in Par. 5.1.2. Three seed samples, labelled CTRL1, CTRL2, CTRL3, were the control treatment of within-population crosses of URB-W, URB-H, CREA. Between-population cross pollination treatments, labelled CR1, CR2, CR2, were made by 3 to 5 sub-treatments (single ramets, Table 8) named IN1-5. Eleven to twenty replicates for each seed sample were prepared according to the amount of seed available.

Seed samples were sown in September 2014 and cultured in a growth chamber (Snijders Economic Deluxe, Thermo-Lab, Codogno, Italy) at 20/10 °C day/night for six months.

Petri dishes were checked monthly for evidence of germination (*sensu* Butcher and Marlow, 1989) and total germination was recorded when no further germination was observed.

Data analysis

Germination data of all sixteen seed samples (3 controls + 3 CR1 + 5 CR2 + 5 CR3) were compared using a Kruskall-Wallis analysis of variance considering firstly the three controls and three crosses as the grouping variable, than considering all the single ramets (i.e. the single seed samples) as the grouping variable. Non parametric ANOVA was chosen because the distribution of germination data was non-normal and thus did not meet the requirements for parametric tests even after arcsin-square root transformation (a large number of replicates had a value of zero).

The analyses were performed using Statistica 8.0 (StatSoft Inc, 2007) on germination data of 262 uncontaminated Petri dishes (anomalous replicates were discarded).

5.2.3. Results

The pollen transfer produced a significant increase in total germination for two populations (CREA, CR2 and URB-H, CR3) but increases were not homogeneous between crosses ($p<0.028$, Table 5 Annex 2) nor between ramets ($p<0.029$, Table 6 Annex 2).

Pollen transfer from the largest population to the smaller ones produced a positive effect only for population CREA, whose mean total germination increased ($p=0.019$) from 1.1% (CTRL2) to 3.1% (CR2), while URB-W (which had the same size and was pollinated by the same donor of CREA) had no significant variation of total germination from the control (2.2 *vs.* 0.9%, $p=0.46$). Cross pollination between small-sized populations (CR3) produced the largest increase in total germination (6.1% *vs.* 1.58%, $p=0.028$). However, the largest differences in total germination occurred between ramets of a single treated population, in all the three crosses (Fig. 10), peaking in one order of magnitude between CR3IN3 and CR3IN5 (13.2% *vs.* 0.4%, $p<0.001$).

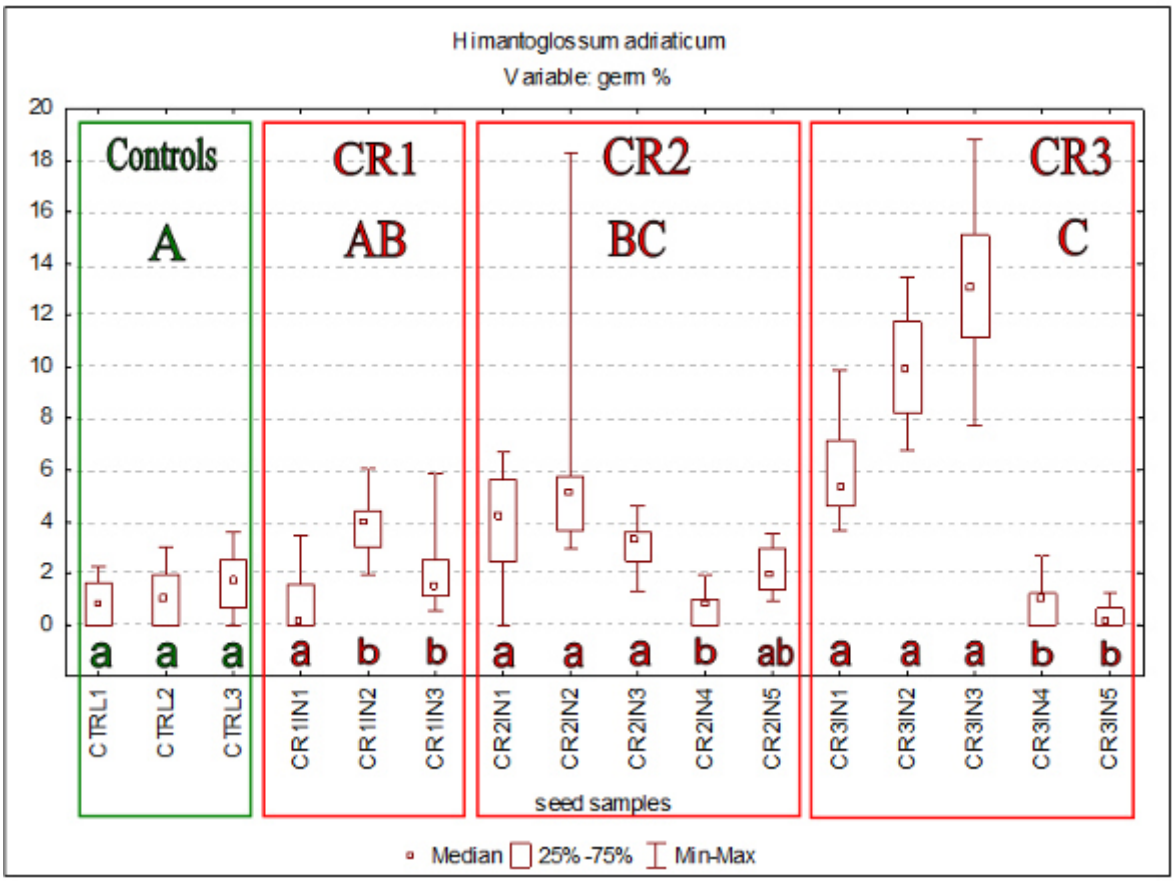

Fig. 10: boxplots of controls (green frame) and treatments (red frame). Different capital letters represent a significant difference between crosses and controls at α=0.05. Different lower case letters represent a significant difference between ramets within crosses at α=0.05.

5.2.4. Discussion

The main result of the test was a significant difference ($p<0.05$, Kruskall-Wallis ANOVA) in germination capacity between ramets within a treatment (e. g. 1 order of magnitude difference), revealing that in the tested population such an attribute of reproductive fitness is basically ramet-specific.

The artificial between-population pollen transfer proved to be effective in enhancing germination capacity of two among the three tested populations. The results were particularly encouraging for the third cross, where the outbreeding was between the smallest but closest populations. In this case the mean increase in germination was almost four times with respect to the germination of the control, while the outbreeding performed on the nearby population using pollen from the largest but most distant population resulted in a doubling of germination with respect to the control.

Thus it can be inferred that inbreeding depression is very likely to occur for populations of this species, even if this conclusion is driven indirectly by the analysis of a fitness-related trait

rather than being directly assessed through analysis of genetic variability (Fay *et al*., 2009; Sletvold *et al*., 2012). It is even possible that outbreeding could have a negative impact on reproductive fitness of the crossed population offspring when adaptive differentiation from selection or chromosomal variant fixation arise between populations (Edmands, 2007). Nevertheless it is extremely unlike to occur in *H. adriaticum* populations since outbreeding depression requires thousands of generations of isolation before starting (Frankham *et al*., 2011).Moreover, between-population outcrossing can certainly be considered as a valuable tool to increase genetic flow also in natural population since the risk of outbreeding depression is much more limited than the risk of inbreeding depression in the case of scarce gene flow (Frankham, 2015).

Additionally, it should be noted that these tests highlighted another important characteristic of *H. adriaticum* germination capacity: it is essentially ramet-specific and mean low germination outcomes are probably the result of a vast majority of poorly performing plant individuals that can produce almost exclusively unviable seeds, and few well-performing individuals with a germination capacity an order of magnitude grater than the average.

5.3. Use of lignin modifying enzymes (LMEs) to aid orchid seed germination

5.3.1. Introduction

It is well known that orchid seeds are generally difficult to germinate in comparison with other taxa since they contain no endosperm and few nutrient reserves to support the development and growth of the plant in the earliest (achlorophyllous) stages (Arditti *et al*., 1981; Ramsay *et al*. 1998). A very small amount of lipids, proteins and rarely starch grains are concentrated in the embryo and may allow the seed to germinate in pure water, but seedlings in nature must be infected by mycorrhizal fungi within a few days in order to get external nutrients and survive (Vermeulen, 1947). However, although mycoheterotrophic symbiosis is of pivotal importance for the nutrient uptake of orchid seedlings and/or adults (Burgeff, 1959), it is not necessarily involved in germination (Knudson, 1922; Rasmussen, 1995) and asymbiotic *in vitro* germination is considered the best available option to propagate orchid species by seed, since the isolation of the proper fungal symbiont is extremely difficult and functions inconsistently (Pierce and Belotti, 2011).

The asymbiotic germination of orchid seeds involves the use an agar base enriched with mineral marco- and micro- nutrients, aminoacids, fruit-derived complex organic media containing phytohormones such as cytokinins that promote cell division of developing embryos (Pierce and Cerabolini, 2011).

Nevertheless, despite of the provision of nutrient and cell-division stimulant compounds to seeds, another important hindrance to orchid germination may occur, since seed dormancy may be present (Knudson, 1950). Several studies (Lee *et al*., 2005; Yamazaki and Miyoshi, 2006; Pierce and Cerabolini, 2011) demonstrated higher germination percentages of immature seeds of terrestrial orchids than mature ones, suggesting they have a morphophysiological dormancy imposed by the formation, late in seed development, of an impermeable seed coat. Yamazaki and Miyoshi (2006) argued that the inner seed integuments of *Cephalanthera falcata*, a terrestrial temperate orchid, undergo a progressive accumulation of lignin and cutin during seed maturation and this process would hinder the embryo growth due to mechanical restriction or chemical reactions. Moreover, periods of cold stratification and the weakening or removal of the seed coat using chemical scarification have proven to prompt germination in several orchid species (e.g.: Rasmussen, 1992; Rasmussen and Wigham, 1993; Wagner and Ansel, 1994; McKendrick *et al*., 2002; Bae *et al*., 2009), highlighting the presence of physical dormacy.

Several useful methods to break orchid seed testa are known, involving soaking seeds in solutions of NaOCl or $Ca(OCl)_2$, H_2SO_4, NaOH, or H_2O_2 (Rasmussen, 1995), freezing-thaw cycles (Pritchard, 1984) or mechanical removal (Butcher and Marlow, 1989). Nevertheless, these treatments are not free from side effects that could be detrimental to orchid seeds, as their testa is composed of only one cell layer in most species and the few cells constituting the small embryo are prone to be suddenly damaged by exposure to the scarifying agent after the testa has been scarified (Aybeke, 2007; Pedroso De Moraes, 2012).

In this study, a new "enzymatic" scarification method was tested, based on the assumption that one of the most common process involved in seed testa weakening in the wild is the degradation of the hardening compounds that make the seed coat sclerotic (phenolic compounds, cutin, suberin, lignin) on the part of white rot fungi. These are ubiquitous fungi responsible for the production of Lignin Modifying Enzymes (LMEs) that digest lignocellulose of rotting woody material in the wild (Hatakka and Hammel, 2010). In the case of affirmative outcome, the use of this technique should be preferred in place of traditional scarification methods using corrosive chemicals, particularly for difficult-to-germinate and threatened species such as *H. adriaticum*, since the enzymes should not be aggressive towards the unlignified cells of the embryo, once the seed testa walls have been broken.The hypothesis to be tested was that the digestion of the seed coat using LMEs aids germination of terrestrial orchid seeds.

5.3.2. Material and methods

Enzyme selection

Three Lignin Modifying Enzymes (LMEs) commercially available (Sigma-Aldrich) were tested on several target species (*Himantoglossum adriaticum*, *Anacamptis morio, Ophrys sphegodes, Ophrys benacensis* and *Cephalanthera longifolia*):

- Laccase from *Pleurotus ostreatus* (LAC);
- Lignin Peroxidase from *Trametes versicolor* (LP);
- Manganese Peroxidase from *Phanerochaete chrysosporium* (MnP).

These are isoenzymes produced by basidiomycetous fungi, classified as nonspecific oxidoreductases with extracellular action (Pollegioni *et al*. 2015). They differ in structure and action: oxidization of non-phenolic lignin substructures into aryl radicals (LP); oxidization of phenolic rings into phenoxy radicals (LAC, MnP) (Hatakka and Hammel, 2010).

In-vitro experiment for hypothesis testing

In an initial experiment the enzyme laccase was administered to seeds of *H. adriaticum* and *A. morio* under sterile conditions *in vitro* on the same Malgrem's modified medium (see Par. 5.1.2. for details), using two methods:

- incorporation of a sterilized enzyme solution directly into the agar substrate;
- bathing the seeds after sowing on the agar surface with the addition of the sterile solution of the enzyme.

In both cases a concentration of 1 unit of active enzyme per seed batch/Petri dish was used.

The sterilization of the enzyme solution in both cases was achieved by cool filtration using a Minisart syringe filter characterized by a pore diameter of 0.2 μm, using a 20 mL syringe.

Given the results of the previous experiments, a second experiment was performed to test the effectiveness of laccase (because it is the most economical enzyme) to aid the germination of the other terrestrial orchid species (*Ophrys sphegodes, Ophrys benacensis, Cephalanthera longifolia*), using the incorporation method. The seed source for all the species but *C. longifolia* were three randomly-chosen seed samples referring to populations tested in Section 5.1.

A third experiment was performed to compare the germination of *A. morio* on substrates containing one of three different LMEs added to the substrate at a concentration of 0.04 U/Petri. This was the highest achievable concentration within cost constraints:while laccase is inexpensive, lignin peroxidase and manganese peroxidase cost up to 300 €/U.

The effectiveness of all three LMEs was tested only for *A. morio* since this is one of the "easiest" terrestrial orchid species to germinate (Pierce and Belotti, 2011) and thus it was considered as a model species. Seeds were sown in March 2014 and stored in the dark in a growth chamber for six months at 20/10°C day/night temperature. Total germination was determined monthly until no new protocorms were detected in the Petri dishes.

Data analysis

For the first and third experiment, between-treatments differences in total germination were assessed by Kruskall-Wallis analysis of variance. A number of 5 to 41 valid germination data records referring to uncontaminated Petri dishes were used for each treatment.

For the second experiment, the comparison between laccase treatments and controls was performed with a Kolmogorov-Smirnov test for two independent samples for each species, using a number of valid data records of 10 to 20.

Analyses were carried out using the "Nonparametrics" tool of Statistica 8.0 software (StatSoft Inc, 2007) on original (non-transformed) data.

5.3.3. Results

Effects of the laccase (1U) – incorporation *vs.* bathing treatment

The final rate of germination for both species was significantly higher than that of the controls (Table 7 Annex 2), only when laccase was added to the substrate (in the case of *H. adriaticum* from 1.3 to 2.3%, $p=0.05$, while for *A. morio* from 23.7 to 49.8%, $p=0.007$; Figs. 11 & 12).
In contrast, the "bathing" treatment significantly reduced germination compared to the control and also introduced contamination (in the case of *H. adriaticum* germination was reduced from 2 to 0.3%; $p<0.001$; while for *A. morio* from 23.7 to 8.5%, $p<0.001$; Figs. 11 & 12).

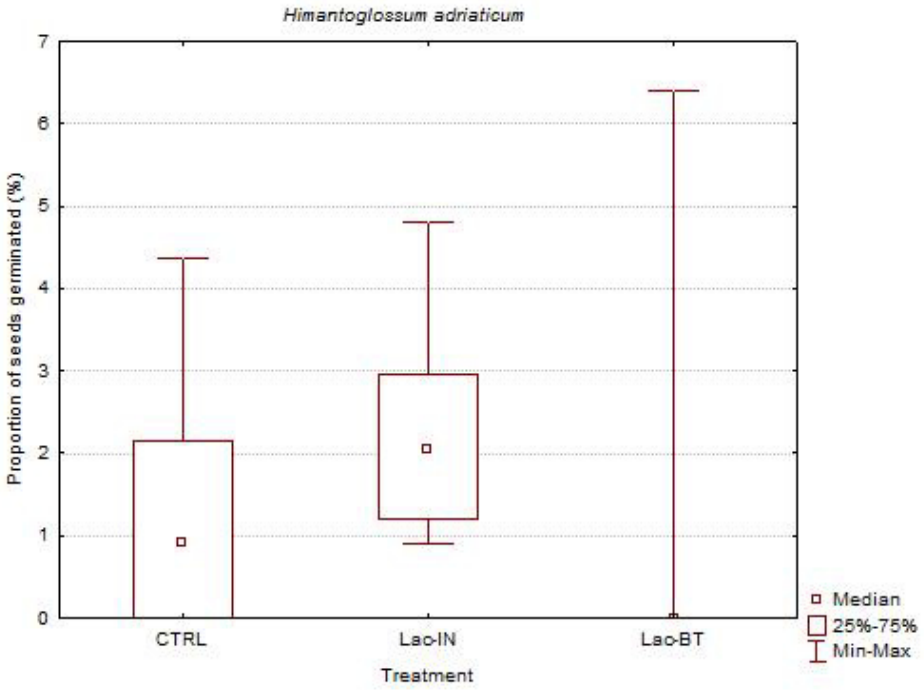

Figure 11: boxplot of treatments of *H. adriaticum*: CTRL=control, Lac-IN=incorporation of laccase in the medium, Lac-BT=bathing the seeds on the medium surface.

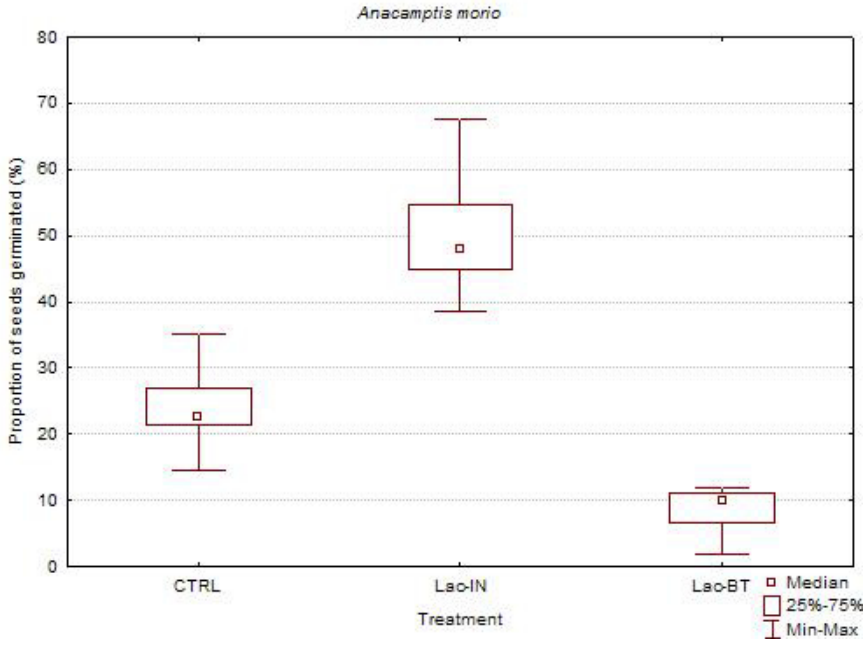

Figure 12: boxplot of treatments of *A. morio*: CTRL=control, Lac-IN=incorporation of laccase in the medium, Lac-BT=bathing the seeds on the medium surface.

Incorporation of laccase in the sowing medium of *O. sphegodes*, *O. benacensis* and *C. longifolia* produced a species-specific response, its effect being significantly positive for one of the three tested species (Fig. 13): the final germination percentage of *O. Benacensis* was doubled (from 9.3 to 19.0 %; $p<0.005$; Table 8 Annex 2). In contrast, no significant differences were detected for

the other two species, despite a slightly greater total germination on treated Petri dishes of *O. sphegodes* (18 *vs*. 13.5%, p>0.10) and an outstanding 6.3% outcome for just one treated Petri dish of *C. longifolia* (0.3 *vs*. 0%, p>0.10).

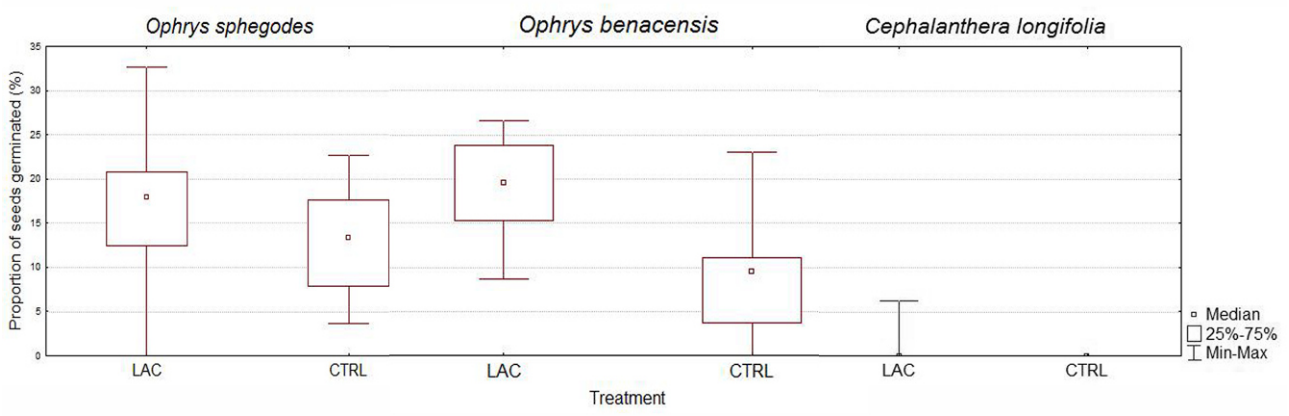

Fig. 13: Boxplots of total germination of laccase (LAC) and control (CTRL) treatments for the three species tested.

Effects of Laccase, Manganese Peroxidase and Lignin Peroxidase (0.04U)

The incorporation of 0.04 U of LMEs in the *A. morio* sowing medium produced a total germination of 28.4% in the case of laccase, 25.8% in the case of lignin peroxidase and 33.0% in the case of manganese peroxidase (Fig. 14), but without significant differences with respect to the control (29.1%, p=0.49, Table 9 Annex 2).

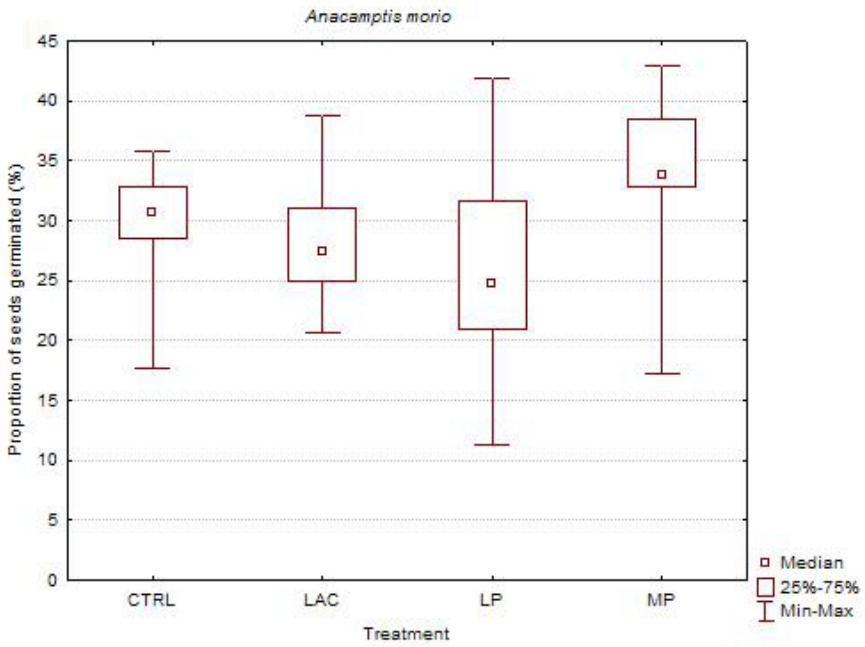

Fig. 14: Boxplots of treatments with three LMEs (CTRL=control, LAC=laccase, LP=lignin peroxidase, MP=manganese peroxidase).

5.3.4. Discussion

The incorporation of laccase in the sowing medium proved to be a significant booster of germination capacity of *A. morio*, *H. adriaticum* and *O. benacensis*. A similar tendency, although not statistically significant, was observed in *O. sphegodes* too. In all the cases the final germination rate was double that of the control and, even if in the case of *H. adriaticum* such an increase meant just 1% more, this may potentially result in an increase of hundreds of plants produced by seeds, since *H. adriaticum* capsules contains tens of thousands seeds.The study found a reliable method for enzyme administration, since the incorporation treatment did not increase the probability of contamination, whilst the bathing treatment did. Actually, the incorporation made the supply of the enzyme comparable to the addition of anything else in the sowing medium recipe, with the only caution of using cool filtration to add the enzyme just after the autoclaved agar medium cooled to 65°C prior to its solidification.

In contrast, lignin peroxidase and manganese peroxidase are too costly to allow useful amounts of enzyme to be applied and as laccase is inexpensive (1 unit of laccase costs three orders of magnitude less than peroxidases), larger amounts can be applied and were found to be effective.
An initial hypothesis regarding the cause of the germination enhancement achieved through the incorporation of laccase was that of the degrading action on the compounds of the orchid seed testa, as previous studies reported an increase in orchid seed germination following a targeted degradation of the seed coat (e.g.: Haervais and Hadley, 1967; Miyoshi and Mii, 1988).

During a placement at the seed morphology lab at the Millennium Seed Bank, Kew (Wakehurst Place, Ardingly, UK), several histochemical tests were performed on *H. adriaticum*, *C. longifolia* and *A. morio* seeds (originating from the populations tested above) to detect the presence of lignin in the seed coat. The Phloroglucinol-HCl method provides a rapid but reliable method to investigate the hypothesis since, whether lignin is present, the tissue exposed to the phloroglucinol will become red-violet in a few minutes due to a specific reaction of lignin functional groups with the dying solution (Gahan, 1984). The test on untreated seeds had a negative outcome and the hypothesis of the presence of lignin had to be rejected.
Subsequently, the findings of Yamazaki and Miyoshi (2006) regarding the supposed presence of lignin in the inner seed coat of *Cephalanthera falcata* and its role in orchid dormancy should be viewed with caution. Perhaps they arrived at the conclusion that the carapace of this species underwent lignification during seed maturation due to the use of safranin as an indicator for lignin. However, although safranin can indicate the presence of lignin, it is not a lignin-specific dye, since it also binds with a wide range of condensed citoplasmatic compounds (Kiviranta *et al*., 1985; Ruzin, 1999).

Furthermore, morphological analysis of untreated and treated seeds of both *H. adriaticum* and *C. longifolia* revealed that the seed coat remained physically unchanged even after exposure to an extremely high activity of 100 U of laccase for 7 days at 20°C (Figs. 15 & 16).

The reasons for which the laccase is able to increase germination still remains unknown and could be of a physiological origin rather than morphological one and must be further investigated.

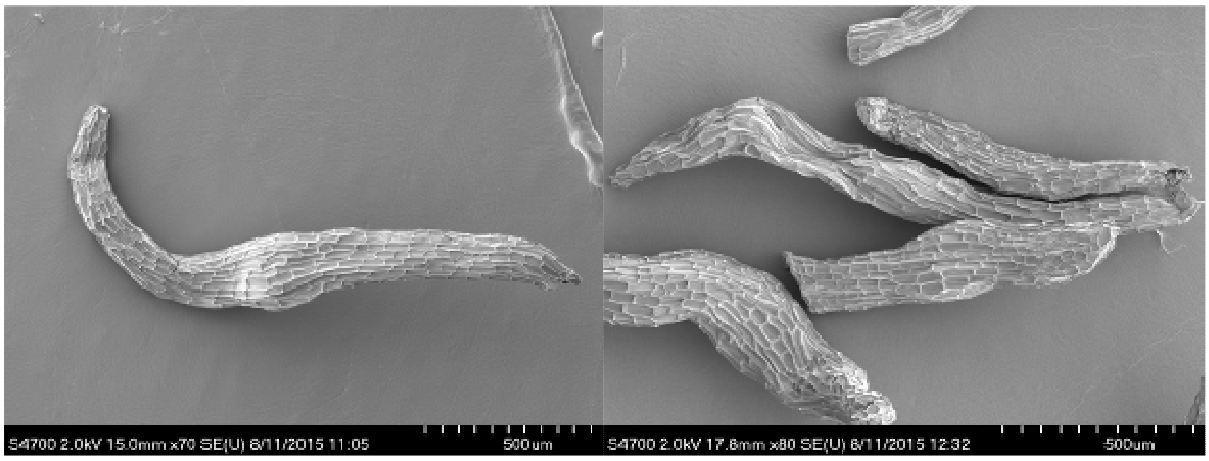

Figure 15: ***Cephalanthera longifolia*** **seeds. SEM microphotographs. Left: control (7 days in distilled H2O + 0.1% Tween 2 surfactant). Right: treatment (7 days in 100 U laccase + 0.1% Tween 2 surfactant).**

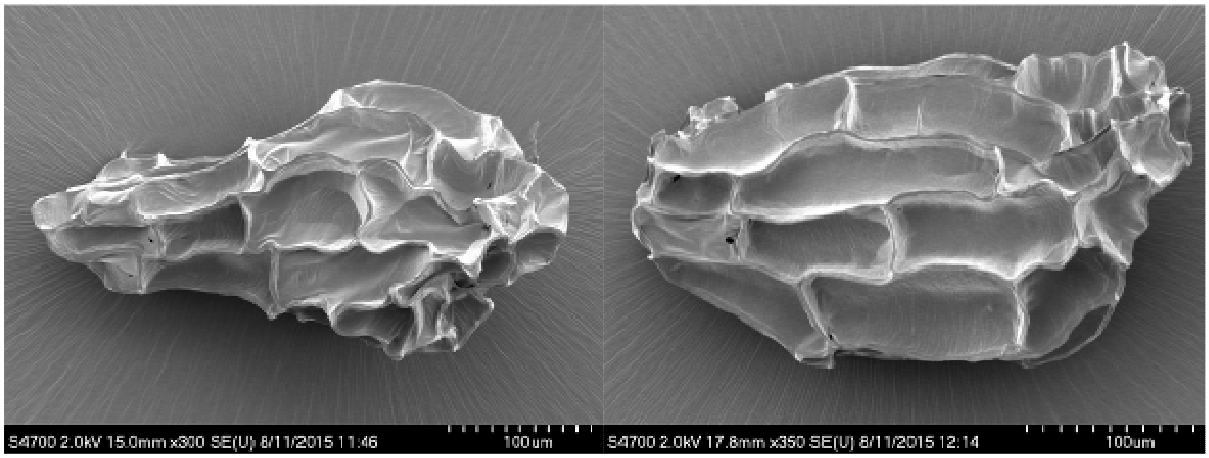

Figure 16: ***Himantoglossum adriaticum*** **seeds. SEM microphotographs. Left: control (7 days in distilled H2O + 0.1% Tween 2 surfactant). Right: treatment (7 days in 100 U laccase + 0.1% Tween 2 surfactant).**

6. General Conclusions

The aim of this work was to investigate which factors influence target orchids distribution, reproductive fitness, the population features (e.g. local density of ramets), and the germination process. Interesting patterns of interaction were found between the reproductive fitness and the structure of the surrounding vegetation. Moreover, local topography and physical soil properties were significant predictors of *H. adriaticum* abundance. Microsite characteristics seems to be very important for the population features and for the germination success. In particular, germination processes are driven by several and interacting factors, that control seed dormancy and viability.

Structural characteristics of surrounding vegetation proved to be the main driver of target orchid fitness and abundance, while the presence of co-flowering entomophilous species seemed not to play a role, despite significant synchronous relationships in flowering periods evident between orchid and non-orchid species.

Reproductive fitness of *H. adriaticum* and *O. sphegodes*, measured as fruit/flower ratio, was favoured where herbaceous vegetation height was lower than orchid flowering stalks, suggesting a possible pollinator-mediated selection on inflorescence height (Walsh *et al*., 2014). This outcome was particularly significant for *H. adriaticum* which displayed a strong positive correlation between the relative height of flowering stalks and the flower fertilization rate. Moreover, the presence of a woody species canopy significantly affected the seed quality of *H. adriaticum*. On the contrary, the fruit/flower ratio of *A. morio* was not affected, perhaps because herbaceous vegetation height and flowering stalk height were both low, and, according to the hypothesis of a pollinator-mediated selection, it is unlikely that selection for inflorescence height occurs in low vegetation (Sltevold *et al*, 2013; Sletvold *et al*., 2014). It is likely that *A. morio* is generally of small stature so that it can avoid the taller herbaceous canopy that develops later in the season and complete its life cycle early.

Indeed, orchid abundance, measured through rosette cover, was negatively affected by herbaceous layer cover, yet not by total vegetation cover or cover of woody species. Moreover, the abundance of *H. adriaticum* was negatively influenced by physical soil properties (namely stoniness and sand content), while it was positively influenced by the topographical features (summarized in the Aridity Index). Since the former soil features are proxies of water availability, while the latter is correlated with a greater surface of bare ground, these findings suggest that the regeneration niche of *H. adriaticum* is constrained by counteracting effects of abiotic harshness, which limits competition with fast-growing species yet, at the same time, imposes a strong selection on seedling survival (Clark *et al*., 2007).

No effects were detected on orchid cover regarding the abundance of blossom types associated with co-flowering non-orchid species: in most cases, entomophilous species flowering

synchronically with orchids did not share the same morphological floral traits. Thus, according to our results, we found no evidence of possible pollinator sharing between orchids and non-orchid species. This could be due the fact that *A. morio* might avoid competition for pollinators with other co-flowering species rather than profit from them for pollinator availability, and could be more targeted at exploiting inexperienced (and more deceivable) pollinators at the beginning of flowering season (i.e. the "remote habitat hypothesis", Lammi and Kuitunen, 1995). *H. adriaticum*, whose flowering period coincides with the flowering peak of the dry grassland, might be independent from co-flowering non-orchid species for pollinator attraction, since its tall and showy inflorescence might act like that of as a magnet species *per se*. *O. sphegodes* relies on a very specialized relationship with male *Andrena* bees that are sexually deceived independently from their foraging behaviour (Schiestl and Ayasse, 2000) and thus may be not sensitive to the presence of co-flowering rewarding species.

On top of this, our findings mostly support the theory of microsite limitation (Eriksson and Ehrlen, 1992) as the main driver of target orchid abundance, according to which orchid species are mostly constrained by the availability of favourable sites that offer space and resources for seed lodging, seedling development, adult plant growth and visibility of the inflorescences towards pollinators. This implies a pivotal role of the almost abandoned management practices that control the vegetation structure, particularly the herbaceous one, but also preserve the topsoil, avoiding severe disturbance.

Indeed, traditional extensive management based on sheep grazing, mowing or haymaking is fundamental to maintain the structure of dry grasslands, preventing colonization by woody species and governing the growth of herbaceous plants. This process promotes ecological opportunities and niche shift or construction within the plant community (Eriksson, 2013), allowing scarcely competitive species, as terrestrial orchids are, to withstand competition with fast-growing species such as those that form clumps or tussocks (Pierce and Belotti, 2011). Many other rare or specialist species may take advantage of weaker competition by herbs that are usually dominant, resulting in a remarkable increase of species richness that makes calcareous dry-grasslands the habitat most rich in plant species within continental Europe (Rolecek *et al*., 2014).

Germination capacity of target orchid seeds was significantly different among contrasting populations, though demographic parameters (population density or number of flowering adults) were not associated with this parameter. For *A. morio* and *O. sphegodes*, not even habitat features were linked to the different outcomes of germination, since populations placed in the same habitats had very different final germination percentages.

On the contrary, a closed vegetation structure (denoted by the presence of a woody species canopy) or an open structure (presence of herbaceous layer only) significantly affected the seed quality of *H. adriaticum*, as seed batches from "closed" plots had a lower germination capacity but an higher proportion of viable embryos with respect to seed batches from "open" plots. This pattern could be explained by a greater visitation rate to flowers by pollinators (evident as greater fruit set) and thus a greater genetic flux in more visible populations positioned in vegetation with an open structure. In open vegetation *H. adriaticum* plants are subjected to a major abiotic stress that probably lower the capability of *H. adriaticum* to produce large amounts of viable seeds (seeds with well-developed embryos).

This hypothesis seems to be corroborated by the evidence of inbreeding depression on isolated populations of *H. adriaticum*. Indeed, where seeds from individual ramets of this species may have a germination capacity greater than 10%, the average germination capacity of seeds collected from several ramets within each populations is only 1.5%. Moreover, outcrossing imposed by artificial pollen transfer allowed a significant increase in total germination of the seed donor populations, especially when the pollen donor population was close to the recipient population, suggesting that seeds of *H. adriaticum* have a very poor germination capacity due to the lack of genetic flux between populations (Herlihy and Eckert, 2002).

Finally, the use of biochemical scarification by mean of the incorporation of lignin modifying enzymes (LMEs) in the culture medium proved to be effective in enhancing germination of *A. morio, H. adriaticum* and *O. benacensis*. Indeed, the final germination of these species doubled when 1 U of laccase was added to the agar solution (before the solidification), using cool sterilization. We also tested the effectiveness of Manganese Peroxidase and Lignin Peroxidase, yet the cost of these enzymes did not allow useful amounts to be employed and no effects were detected at the low activity that could be tested (0.04 U). Contrary to expected, on the base of a previous study (Yamazaki and Myoshi, 2006), lignin was not found in the seed testa of target orchids, thus the supposed effectiveness of laccase due to the digestion of lignin and subsequent removal of morphological dormancy was discarded as the possible explanation and further research is needed to find the cause. In any case, the use of laccase in promoting orchid seed germination seems highly promising since it avoids the potentially lethal side-effects on embryos posed by the traditional scarification techniques and might be successfully applied to the conservation of other dust-seed species (e.g. *Orobancaceae, Ericaceae* or some rare species of *Gentianaceae*).

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- *www.coe.int/t/dg4/cultureheritage/nature/Bern* (Convention on the conservation of European wildlife and natural habitats – Convention of Bern);
- *www.biodiv.org* (Convention on Biological Diversity - CBD);
- *http://www.arpa.veneto.it/bollettini/htm/dati_idrometeo.asp* (climatic data for the study area);
- *http://idt.regione.veneto.it//app/metacatalog/* (Land Use Cartography (Corin Land Cover 2006 Legend) of the Veneto Region).

ANNEX 1

Table 1: Independent (soil) and dependent (orchid fitness) variables referring to tested *H. adriaticum* populations

Population ID code	SS (%)	GR (%)	CL (%)	SN (%)	ST (%)	NO3- (mg/Kg)	PO4-3 (mg/Kg)	CO3-2 (g/Kg)	pH	CO (g/Kg)	AI	PPLA (cm^2)	PFSH (cm)	OrcDen
36	14.7	68.8	5.4	54.2	40.3	8.7	1.4	292	7.8	75.2	125	133.3	66.3	115
37	0.0	38.7	3.8	66.8	29.3	19.5	3.7	175.0	7.5	51.9	1500	62.7	50.0	96
38	0.0	47.8	8.1	60.6	31.3	17.3	1.8	286.2	8.0	93.1	875	138.8	69.8	13
41	1.9	11.2	6.6	59.6	33.8	11.9	4.5	164.1	7.7	86.4	500	118.4	56.2	8
42	0.8	41.8	5.2	56.6	38.2	12.6	4.7	257.7	7.9	71.6	200	118.8	50.7	59
45	21.1	81.4	5.5	69.8	24.6	30.2	7.4	177.7	7.3	96.1	125	82.2	42.6	15
46	0.0	30.6	3.9	46.5	49.5	8.7	3.4	85.5	7.4	65.0	1125	87.9	61.8	62
47	0.0	30.8	5.2	59.0	35.8	12.6	3.0	278.1	7.5	72.2	150	152.8	67.5	13
48	0.0	33.8	7.4	57.4	35.1	9.1	3.7	295.7	7.5	37.6	750	69.0	50.3	9
49	0.0	19.6	5.3	44.4	50.3	16.9	3.9	40.7	7.8	102.9	1500	84.4	62.8	26
50	22.2	45.5	3.1	63.2	33.6	27.2	5.5	251.0	7.8	114.2	875	165.7	71.2	15
51	3.7	49.6	4.6	70.2	25.2	15.3	4.8	221.1	7.9	52.4	5250	30.2	41.6	123
52	0.0	37.9	2.4	63.5	34.1	15.5	5.0	240.1	7.9	46.3	1000	66.3	57.4	11
53	7.4	38.5	3.0	45.7	51.3	13.1	5.1	116.7	7.9	49.1	3125	65.9	53.6	26
54	8.9	25.1	6.5	62.5	31.0	18.1	5.1	229.3	7.8	157.7	1250	121.8	66.0	23
55	14.0	45.0	5.1	64.4	30.5	24.4	3.6	226.5	8.0	28.3	4375	61.1	54.1	11
56	4.3	33.0	2.2	64.7	33.0	14.7	4.9	221.1	7.9	32.7	1750	43.5	52.0	101
57	36.8	74.7	5.4	66.7	27.9	11.9	7.0	181.8	7.8	121.4	625	78.6	69.2	9
58	4.0	10.2	4.8	40.9	54.3	24.6	5.7	275.4	7.9	25.5	4375	44.1	42.9	37
59	10.0	29.5	5.5	37.0	57.5	11.4	3.5	267.2	7.9	93.1	1000	86.9	61.8	28

Table 2: Result of Principal Component Analysis. Eigenvalues of correlation matrix

Value number	Eigenvalue	% Total Variance	Cumulative Eigenvalue	Cumulative %
1	3.45	31.39	3.45	31.39
2	1.91	17.39	5.37	48.78
3	1.66	15.08	7.03	63.87
4	1.22	11.09	8.24	74.95
5	0.79	7.16	9.03	82.11
6	0.70	6.39	9.74	88.50
7	0.57	5.15	10.30	93.65
8	0.39	3.59	10.70	97.24
9	0.18	1.61	10.87	98.84
10	0.13	1.16	11.00	100.00

Table 3: Result of Principal Component Analysis.
Factor coordinates of the variables, based on correlations (factor loadings) and contributions of variables to factors (principal components). Marked loadings are grater than 0.7.

Variables	Factor Loadings		Variable contributions	
	Factor 1	Factor 2	Factor 1	Factor 2
Stoniness (%)	**-0.74**	-0.12	0.16	0.01
Gravel (%)	**-0.83**	-0.04	0.20	0.00
Sand (%)	**-0.79**	-0.07	0.18	0.00
Silt (%)	**0.78**	-0.04	0.18	0.00
Clay (%)	-0.03	0.66	0.00	0.22
NO_3^- (mg/Kg)	-0.49	-0.43	0.07	0.10
PO_4^{3-} (mg/Kg)	-0.64	-0.32	0.12	0.05
CO_3^{2-} (g/Kg)	-0.08	0.01	0.00	0.00
pH	0.30	-0.43	0.03	0.10
Organic Carbon (g/Kg)	-0.40	0.58	0.05	0.18
Aridity Index	0.23	**-0.81**	0.02	0.34

Table 4: Spearman Rank Order Correlations of selected soil variables.
Marked correlations are significant at p <0.05

Variables	Stoniness (%)	Gravel (%)	Sand (%)	Silt (%)	Aridity Index
Stoniness (%)	1.00	0.33	0.26	-0.30	0.04
Gravel (%)	0.33	1.00	**0.69**	**-0.69**	-0.18
Sand (%)	0.26	**0.69**	1.00	**-0.95**	-0.01
Silt (%)	-0.30	**-0.69**	**-0.95**	1.00	0.07
Aridity Index	0.04	-0.18	-0.01	0.07	1.00

ANNEX 2

Table 1: Kruskal-Wallis ANOVA by Ranks of *A. morio* populations: whole model (first window), Multiple Comparisons z' values (second window) and Multiple Comparisons 2-tailed p values (third window).

Kruskal-Wallis ANOVA by Ranks;
Proportion of seeds germinated (%)
Independent (grouping) variable: Population
Kruskal-Wallis test: H (d.f. 4, N= 49) =36.67 p = <0.0001

Population ID code	Code	Number of valid cases	Sum of ranks
26	1	10	165.5
30	2	10	384
31	3	9	277
34	4	10	55
44	5	10	343.5

Multiple Comparisons z' values;
Proportion of seeds germinated (%)
Independent (grouping) variable: Population
Kruskal-Wallis test: H (d.f. 4, N= 49) =36.67 p = <0.0001

Population ID code	26	30	31	34	44
26		**3,42**	2,17	1,73	**2,79**
30	**3,42**		1,16	**5,15**	0,63
31	2,17	1,16		**3,85**	0,54
34	1,73	**5,15**	**3,85**		**4,51**
44	**2,79**	0,63	0,54	4,51	

Multiple Comparisons p values (2-tailed);
Proportion of seeds germinated (%)
Independent (grouping) variable: Population
Kruskal-Wallis test: H (d.f. 4, N= 49) =36.67 p = <0.0001

Population ID code	26	30	31	34	44
26		**0,0063**	0,3022	0,8377	**0,0534**
30	**0,0063**		1,0000	**0,0000**	1,0000
31	0,3022	1,0000		**0,0012**	1,0000
34	0,8377	**<0.0001**	**0,0012**		**<0,0001**
44	**0,0534**	1,0000	1,0000	**<0,0001**	

Table 2: Kruskal-Wallis ANOVA by Ranks of *O. shegodes* populations: whole model (first window), Multiple Comparisons z' values (second window) and Multiple Comparisons 2-tailed p values (third window).

Kruskal-Wallis ANOVA by Ranks;
Proportion of seeds germinated (%)
Independent (grouping) variable: Population
Kruskal-Wallis test: H (d.f.4, N= 69) =47.05 p = <0.0001

Population ID code	Code	Number of valid cases	Sum of ranks
7	1	20	444.5
13	2	19	956
21	3	10	546
23	4	10	81
29	5	10	387.5

Multiple Comparisons z' values;
Proportion of seeds germinated (%)
Independent (grouping) variable: Population
Kruskal-Wallis test: H (d.f. 4, N= 69) =47.05 p = <0.0001

Population ID code	7	13	21	23	29
7		**4,37**	**4,17**	1,82	**2,13**
13	**4,37**		0,55	**5,39**	1,48
21	**4,17**	0,55		**5,18**	1,77
23	1,82	**5,39**	**5,18**		**3,42**
29	2,13	1,48	1,77	**3,42**	

Multiple Comparisons p values (2-tailed);
Proportion of seeds germinated (%)
Independent (grouping) variable: Population
Kruskal-Wallis test: H (d.f. 4, N= 69) =47.05 p = <0.0001

Population ID code	7	13	21	23	29
7		**0,0001**	**0,0003**	0,6909	**0,3344**
13	**0,0001**		1,0000	**<0.0001**	1,0000
21	**0,0003**	1,0000		**<0.0001**	0,7730
23	0,6909	**<0.0001**	**<0.0001**		**0,0064**
29	0,3344	1,0000	0,7730	**0,0064**	

Table 3: Kruskal-Wallis ANOVA by Ranks of *H. adriaticum* populations: whole model (first window), Multiple Comparisons z' values (second window) and Multiple Comparisons 2-tailed p values (third window).

Kruskal-Wallis ANOVA by Ranks;
Proportion of seeds germinated (%)
Independent (grouping) variable: Population
Kruskal-Wallis test: H (d.f.9, N= 195) =36.05 p = <0.0001

Population ID code	Code	Valid N	Sum of ranks
36	1	22	2708,50
41	2	22	1342,00
45	3	20	2897,00
46	4	20	1940,50
47	5	20	2190,50
50	6	21	1545,00
51	7	17	1809,00
57	8	18	1610,50
58	9	17	1562,00
59	10	18	1505,00

Multiple Comparisons p values (2-tailed);
Proportion of seeds germinated (%)
Independent (grouping) variable: Population
Kruskal-Wallis test: H (d.f. 9, N= 195) =36.05 p = <0.0001

Population ID code	36	41	45	46	47	50	51	57	58	59
36		**0,01**	1,00	1,00	1,00	0,18	1,00	1,00	1,00	1,00
41	**0,01**		**<0.0001**	1,00	0,24	1,00	0,57	1,00	1,00	1,00
45	1,00	**<0.0001**		0,33	1,00	**<0.0001**	1,00	0,11	0,20	**0,04**
46	1,00	1,00	0,33		1,00	1,00	1,00	1,00	1,00	1,00
47	1,00	0,24	1,00	1,00		1,00	1,00	1,00	1,00	1,00
50	0,18	1,00	**<0.0001**	1,00	1,00		1,00	1,00	1,00	1,00
51	1,00	0,57	1,00	1,00	1,00	1,00		1,00	1,00	1,00
57	1,00	1,00	0,11	1,00	1,00	1,00	1,00		1,00	1,00
58	1,00	1,00	0,20	1,00	1,00	1,00	1,00	1,00		1,00
59	1,00	1,00	**0,04**	1,00	1,00	1,00	1,00	1,00	1,00	

Multiple Comparisons z' values;
Proportion of seeds germinated (%)
Independent (grouping) variable: Population
Kruskal-Wallis test: H (d.f.9, N= 195) =36.05 p = <0.0001

Population ID code	36	41	45	46	47	50	51	57	58	59
36		**3,6503**	1,2466	1,4962	0,7793	2,8774	0,9165	1,8756	1,7137	2,2024
41	**3,6503**		**4,8089**	2,0661	2,7830	0,7302	2,4918	1,5874	1,6946	1,2606
45	1,2466	**4,8089**		2,6798	1,9794	**4,0424**	2,0647	3,0202	2,8451	**3,3399**
46	1,4962	2,0661	2,6798		0,7004	1,3301	0,5042	0,4119	0,2762	0,7316
47	0,7793	2,7830	1,9794	0,7004		2,0390	0,1672	1,0937	0,9476	1,4133
50	2,8774	0,7302	**4,0424**	1,3301	2,0390		1,7836	0,8772	0,9945	0,5538
51	0,9165	2,4918	2,0647	0,5042	0,1672	1,7836		0,8875	0,7506	1,1946
57	1,8756	1,5874	3,0202	0,4119	1,0937	0,8772	0,8875		0,1263	0,3116
58	1,7137	1,6946	2,8451	0,2762	0,9476	0,9945	0,7506	0,1263		0,4334
59	2,2024	1,2606	**3,3399**	0,7316	1,4133	0,5538	1,1946	0,3116	0,4334	

Table 4: Kolmogorow-Smirnov test for differences between two groups populations: open ("A") vs. closed ("C") vegetation structure.

Population	Max Neg	Max Pos	p-level	Mean "A population"	Mean "C population"	S.D. "A"	S.D. "C"	Number of valid cases "A"	Number of valid cases "C"
Proportion of seeds germinated (%)	0.00	0.217	< 0.05	1.8	1.1	2.06	1.25	84	94
Proportion of embryos germinated (%)	0.00	0.356	< 0.001	3.4	1.4	3.92	1.54	84	94
Proportion of unfertile seeds (%)	0.00	0.829	< 0.001	43.1	20.1	9.16	8.58	84	94

Table 5: Kruskal-Wallis ANOVA by Ranks of *H. adriaticum* cross-pollinated populations: whole model (first window), Multiple Comparisons z' values (second window) and Multiple Comparisons 2-tailed p values (third window).

Kruskal-Wallis ANOVA by Ranks;
Proportion of seeds germinated (%)
Independent (grouping) variable: controls and crosses
Kruskal-Wallis test: H (d.f. 5, N= 262) =43,64 p = <0.0001

Population ID code	Code	Number of valid cases	Sum of Ranks
CTRL1	101	20	1463.5
CTRL2	102	20	1564.5
CTRL3	103	19	2002
CR1	104	48	5601
CR2	105	71	9944.5
CR3	106	84	13877.5

Multiple Comparisons z' values;
Proportion of seeds germinated (%)
Independent (grouping) variable: controls and crosses
Kruskal-Wallis test: H (d.f.5, N= 262) =43,64 p = <0.0001

Population ID code	CTRL1	CTRL2	CTRL3	CR1	CR2	CR3
CTRL1		0,21	1,33	**2,16**	**3,49**	**4,88**
CTRL2	0,21		1,12	**1,91**	**3,22**	**4,61**
CTRL3	1,33	1,12		0,55	1,77	**3,11**
CR1	2,16	1,91	0,55		1,65	**3,54**
CR2	**3,49**	**3,22**	1,77	1,65		2,06
CR3	**4,88**	**4,61**	**3,11**	**3,54**	2,06	

Multiple Comparisons p values (2-tailed);
Proportion of seeds germinated (%)
Independent (grouping) variable: controls and crosses
Kruskal-Wallis test: H (d.f.5, N= 262) =43,64 p = <0.0001

Population	CTRL1	CTRL2	CTRL3	CR1	CR2	CR3
CTRL1		1,0000	1,0000	**0,4645**	**0,0073**	**<0.0001**
CTRL2	1,0000		1,0000	**0,8476**	**0,0190**	**0,0001**
CTRL3	1,0000	1,0000		1,0000	1,0000	**0,0282**
CR1	0,4645	0,8476	1,0000		1,0000	**0,0060**
CR2	**0,0073**	**0,0190**	1,0000	1,0000		0,5934
CR3	**<0.0001**	**0,0001**	**0,0282**	**0,0060**	0,5934	

Table 6: Kruskal-Wallis ANOVA by Ranks of *H. adriaticum* cross-pollinated ramets: whole model (first window), Multiple Comparisons z' values (second window) and Multiple Comparisons 2-tailed p values (third window).

Kruskal-Wallis ANOVA by Ranks;
Proportion of seeds germinated (%)
Independent (grouping) variable: seed sample
Kruskal-Wallis test: H (d.f.15, N= 262) =196,53 p = <0.0001

Treatment	Code	Number of valid cases	Sum of Ranks
CTRL1	101	20	1464
CTRL2	102	20	1565
CTRL3	103	19	2002
CR1IN1	104	19	1228
CR1IN2	105	14	2547
CR1IN3	106	15	1826
CR2IN1	107	11	1783
CR2IN2	108	14	2822
CR2IN3	109	15	2410
CR2IN4	110	16	986
CR2IN5	111	15	1944
CR3IN1	112	20	4221
CR3IN2	113	15	3587
CR3IN3	114	16	4009
CR3IN4	115	18	1362
CR3IN5	116	15	699

Multiple Comparisons z' values;
Proportion of seeds germinated (%)
Independent (grouping) variable: seed sample
Kruskal-Wallis test: H (d.f.15, N= 262) =196,53 p = <0.0001

Treatment	CTRL1	CTRL2	CTRL3	CR1IN1	CR1IN2	CR1IN3	CR2IN1	CR2IN2	CR2IN3	CR2IN4	CR2IN5	CR3IN1	CR3IN2	CR3IN3	CR3IN4	CR3IN5
CTRL1		0,21	1,33	0,35	**4,12**	1,88	3,13	**4,86**	3,38	0,45	2,18	**5,75**	**6,41**	**6,98**	0,10	1,03
CTRL2	0,21		1,12	0,56	**3,93**	1,68	2,95	**4,67**	3,19	0,65	1,98	**5,54**	**6,22**	**6,78**	0,10	1,22
CTRL3	1,33	1,12		1,66	2,87	0,63	1,98	**3,60**	2,11	1,70	0,93	**4,35**	**5,11**	**5,65**	1,19	2,25
CR1IN1	0,35	0,56	1,66		**4,39**	2,18	3,39	**5,13**	**3,67**	0,12	2,48	**6,03**	**6,67**	**7,23**	0,44	0,69
CR1IN2	**4,12**	**3,93**	2,87	**4,39**		2,14	0,65	0,68	0,76	**4,34**	1,86	1,10	2,03	2,47	**3,94**	**4,81**
CR1IN3	1,88	1,68	0,63	2,18	2,14		1,34	2,83	1,41	2,21	0,28	3,45	**4,24**	**4,73**	1,74	2,72
CR2IN1	3,13	2,95	1,98	3,39	0,65	1,34		1,29	0,05	3,38	1,08	1,72	2,56	2,98	2,98	**3,84**
CR2IN2	**4,86**	**4,67**	**3,60**	**5,13**	0,68	2,83	1,29		1,45	**5,05**	2,55	0,36	1,34	1,77	**4,66**	**5,50**
CR2IN3	3,38	3,19	2,11	**3,67**	0,76	1,41	0,05	1,45		**3,64**	1,12	1,95	2,84	3,30	3,21	**4,12**
CR2IN4	0,45	0,65	1,70	0,12	**4,34**	2,21	3,38	**5,05**	**3,64**		2,50	**5,88**	**6,52**	**7,05**	0,54	0,55
CR2IN5	2,18	1,98	0,93	2,48	1,86	0,28	1,08	2,55	1,12	2,50		3,15	**3,96**	**4,44**	2,04	3,00
CR3IN1	**5,75**	**5,54**	**4,35**	**6,03**	1,10	3,45	1,72	0,36	1,95	**5,88**	3,15		1,09	1,55	**5,50**	**6,35**
CR3IN2	**6,41**	**6,22**	**5,11**	**6,67**	2,03	**4,24**	2,56	1,34	2,84	**6,52**	**3,96**	1,09		0,42	**6,17**	**6,96**
CR3IN3	**6,98**	**6,78**	**5,65**	**7,23**	2,47	**4,73**	2,98	1,77	3,30	**7,05**	**4,44**	1,55	0,42		**6,72**	**7,49**
CR3IN4	0,10	0,10	1,19	0,44	**3,94**	1,74	2,98	**4,66**	3,21	0,54	2,04	**5,50**	**6,17**	**6,72**		1,10
CR3IN5	1,03	1,22	2,25	0,69	**4,81**	2,72	**3,84**	**5,50**	**4,12**	0,55	3,00	**6,35**	**6,96**	**7,49**	1,10	

Multiple Comparisons p values (2-tailed);
Proportion of seeds germinated (%)
Independent (grouping) variable: seed sample
Kruskal-Wallis test: H (d.f.15, N= 262) =196,53 p = <0.001

Treatment	CTRL1	CTRL2	CTRL3	CR1IN1	CR1IN2	CR1IN3	CR2IN1	CR2IN2	CR2IN3	CR2IN4	CR2IN5	CR3IN1	CR3IN2	CR3IN3	CR3IN4	CR3IN5
CTRL1		1.000	1.000	1.000	**0.005**	1.000	0.213	**0.000**	0.087	1.000	1.000	**0.000**	**0.000**	**0.000**	1.000	1.000
CTRL2	1.000		1.000	1.000	**0.010**	1.000	0.383	**0.000**	0.174	1.000	1.000	**0.000**	**0.000**	**0.000**	1.000	1.000
CTRL3	1.000	1.000		1.000	0.495	1.000	1.000	**0.038**	1.000	1.000	1.000	**0.002**	**0.000**	**0.000**	1.000	1.000
CR1IN1	1.000	1.000	1.000		**0.001**	1.000	0.082	**0.000**	**0.029**	1.000	1.000	**0.000**	**0.000**	**0.000**	1.000	1.000
CR1IN2	**0.005**	**0.010**	0.495	**0.001**		1.000	1.000	1.000	1.000	**0.002**	1.000	1.000	1.000	1.000	**0.010**	**0.000**
CR1IN3	1.000	1.000	1.000	1.000	1.000		1.000	0.552	1.000	1.000	1.000	0.067	**0.003**	**0.000**	1.000	0.792
CR2IN1	0.213	0.383	1.000	0.082	1.000	1.000		1.000	1.000	0.085	1.000	1.000	1.000	0.345	0.346	**0.015**
CR2IN2	**0.000**	**0.000**	**0.038**	**0.000**	1.000	0.552	1.000		1.000	**0.000**	1.000	1.000	1.000	1.000	**0.000**	**0.000**
CR2IN3	0.087	0.174	1.000	**0.029**	1.000	1.000	1.000	1.000		**0.033**	1.000	1.000	0.549	0.116	0.160	**0.004**
CR2IN4	1.000	1.000	1.000	1.000	**0.002**	1.000	0.085	**0.000**	**0.033**		1.000	**0.000**	**0.000**	**0.000**	1.000	1.000
CR2IN5	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		0.198	**0.009**	**0.001**	1.000	0.323
CR3IN1	**0.000**	**0.000**	**0.002**	**0.000**	1.000	0.067	1.000	1.000	1.000	**0.000**	0.198		1.000	1.000	**0.000**	**0.000**
CR3IN2	**0.000**	**0.000**	**0.000**	**0.000**	1.000	**0.003**	1.000	1.000	0.549	**0.000**	**0.009**	1.000		1.000	**0.000**	**0.000**
CR3IN3	**0.000**	**0.000**	**0.000**	**0.000**	1.000	**0.000**	0.345	1.000	0.116	**0.000**	**0.001**	1.000	1.000		**0.000**	**0.000**
CR3IN4	1.000	1.000	1.000	1.000	**0.010**	1.000	0.346	**0.000**	0.160	1.000	1.000	**0.000**	**0.000**	**0.000**		1.000
CR3IN5	1.000	1.000	1.000	1.000	**0.000**	0.792	**0.015**	**0.000**	**0.004**	1.000	0.323	**0.000**	**0.000**	**0.000**	1.000	

Table 7: Kruskal-Wallis ANOVA by Ranks of *H. adriaticum* and *A. morio* treatments with laccase: whole model (first window), Multiple Comparisons z' values (second window) and Multiple Comparisons 2-tailed p values (third window). CTRL=control, Lac-IN=incorporation of laccase in the medium, Lac-BT=bathing the seeds on the medium surface.

Himantoglossum adriaticum Kruskal-Wallis test: H (d.f. 2, N= 85) =41.97 p =< 0.0001				*Anacamptis morio* Kruskal-Wallis test: H (d.f. 2, N= 35) =30.00 p = <0.0001			
Treatment	Code	Valid number of cases	Sum of Ranks	Treatment	Code	Valid number of cases	Sum of Ranks
CTRL	101	24	1193	CTRL	101	14	245
Lac-IN	102	19	1278,5	Lac-IN	102	11	330
Lac-BT	103	42	1183,5	Lac-BT	103	10	55
Multiple Comparisons z' values				Multiple Comparisons z' values			
Treatment	CTRL	Lac-IN	Lac-BT	Treatment	CTRL	Lac-IN	Lac-BT
CTRL		**2,32**	**3,41**	CTRL		**3,03**	**2,83**
Lac-IN	**2,32**		**5,73**	Lac-IN	**3,03**		**5,47**
Lac-BT	**3,41**	**5,73**		Lac-BT	**2,83**	**5,47**	
Multiple Comparisons p values (2-tailed)				Multiple Comparisons p values (2-tailed)			
Treatment	CTRL	Lac-IN	Lac-BT	Treatment	CTRL	Lac-IN	Lac-BT
CTRL		**0,0611**	**0,0020**	CTRL		**0,0074**	**0,0140**
Lac-IN	**0,0611**		**<0,0001**	Lac-IN	**0,0074**		**<0,0001**
Lac-BT	**0,0020**	**<0,0001**		Lac-BT	**0,0140**	**<0,0001**	

Table 8: Results of Kolmogorov-Smirnov test for differences between treatments. LAC=laccase incorporation CTRL=control.

Specie	Max negative differnce	Max positive differnce	p-level	Mean LAC	Mean CTRL	S. D. LAC	S. D. CTRL	Number of valid cases LAC	Number of valid cases CTRL
O. sphegodes	0.000	0.319	> 0.10	18.0	13.5	8.348	5.880	20	13
O. benacensis	0.000	0.695	<0 .005	19.0	9.3	5.552	6.559	19	10
C. longifolia	0.000	0.053	> 0.10	0.3	0.0	1.434	0.000	19	13

Table 9: Kruskal-Wallis ANOVA by Ranks *A. morio* treatments with laccase (LAC), lignin peroxidase (LP) and manganese peroxidase (MP): whole model (first window), Multiple Comparisons z' values (second window) and Multiple Comparisons 2-tailed p values (third window). CTRL=control.

Kruskal-Wallis ANOVA by Ranks;
Proportion of seeds germinated (%)
Independent (grouping) variable: Treatment
Kruskal-Wallis test: H (d.f.3, N= 22) =2.43 p =.4869

Treatment	Code	Valid	Sum of
CTRL	101	5	58
LAC	102	6	63
LP	103	6	56
MP	104	5	76

Multiple Comparisons z' values;
Proportion of seeds germinated (%)
Independent (grouping) variable: Treatment
Kruskal-Wallis test: H (d.f.3, N= 22) =2.43 p =.4869

Treatment	CTRL	LAC	LP	MP
CTRL		0,28	0,58	0,88
LAC	0,28		0,31	1,20
LP	0,58	0,31		1,49
MP	0,88	1,20	1,49	

Multiple Comparisons p values (2-tailed);
Proportion of seeds germinated (%)
Independent (grouping) variable: Treatment
Kruskal-Wallis test: H (d.f. 3, N= 22) =2.43 p =.4869

Treatment	CTRL	LAC	LP	MP
CTRL		1,0000	1,0000	1,0000
LAC	1,0000		1,0000	1,0000
LP	1,0000	1,0000		0,8142
MP	1,0000	1,0000	0,8142	

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Estratto per riassunto della tesi di dottorato

Studente: ***ANTONIO SLAVIERO*** ***matricola: 830911***
Dottorato: ***Scienze Ambientali***
Ciclo: 28^

Titolo della tesi[1] *:* ***Ecology and conservation strategies of target dry grassland orchid species (Orchidaceae)***

Abstract

Due to their high degree of ecological specialisation, orchids (*Orchidacea*) are vulnerable species, threatened at the local and global level, yet also bear a value as bioindicators of ecosystem health. Since the classical species-based approach for the biodiversity conservation has left place to a more holistic approach, improving the scientific knowledge of the species ecology is an essential tool to target their conservation. This thesis considered three target species (*A. morio, H. adriaticum, O. sphegodes*) of semi-natural calcareous dry-grassland and analyzed the effect of the vegetation structural features, the relationships with pollinators, local topography and soil properties on the orchid distribution and fitness. Results support the microsite limitation theory as the main driver of orchid abundance and fitness. The germination capacity of seeds from contrasting populations was taken into account as well to study the effect of artificial pollen transfer on inbreeding of isolated populations and a new biochemical method to scarify seed testa has been proposed.

Riassunto

A causa del loro elevato livello di specializzazione ecologica, le *Orchidaceae* sono specie altamente vulnerabili e minacciate a scala globale ma anche bioindicatori dello stato degli habitat in cui vivono. Al fine di salvaguardare la biodiversità si rende necessario un approccio conservativo basato sulla conoscenza dell'ecologia delle specie, degli habitat in cui vivono e dei rapporti con altri organismi. Questa tesi considera tre orchidee (*A. morio; H. adriaticum, O. sphegodes*) quali target di studio per comprendere quali fattori ecologici siano i driver della loro fitness in un habitat particolarmente ricco di specie ma anche minacciato a livello europeo, la prateria arida semi-naturale su substrato calcareo. Vengono analizzati la struttura della vegetazione, i rapporti con i pronubi, la topografia locale e l'ambiente edafico, ottenendo interessanti pattern rispetto alla copertura e fitness riproduttiva delle specie target. Un approfondimento è dedicato ai processi di germinazione evidenziando l'utilità dell'impollinazione artificiale per superare l'inbreeding e testando un nuovo metodo di scarificazione enzimatica.

Firma dello studente

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[1] Il titolo deve essere quello definitivo, uguale a quello che risulta stampato sulla copertina dell'elaborato consegnato.