Reproductive biology of *Angraecum eburneum* subsp. *superbum* (Orchidaceae) under glasshouse conditions

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Abstract

The results of long-term observations on the reproductive biology of an epiphytic monopodial orchid, *Angraecum eburneum* subsp. *superbum*, under glasshouse conditions are provided. Experimental pollination treatments (induced autogamy/induced xenogamy) indicated that *A. eburneum* subsp. *superbum* is a self-compatible but not an autogamous orchid. The fruit set after self- and cross-pollination significantly differed. Cross-pollination had positive effect on seed viability of *A. eburneum* subsp. *superbum* under glasshouse conditions. The fruit set, seed mass and seed viability were compared with respect to flower position within an inflorescence. The position of pollinated flowers within the inflorescence was found to affect the fruit and seed mass, but not seed viability. The impact of various factors, including breeding systems, pollination strategies, flowering longevity, pollinated flower position in inflorescence and resource status, upon reproductive success of the orchid studied *ex situ* was analyzed.

Key words: *Angraecum eburneum* subsp. *superbum*, cross-pollination, flowering phenology, fruit set, morphometry, self-pollination, self-compatibility, seed viability.
Abbreviations: ISI, self-incompatibility index.

Introduction

Ex situ conservation of tropical orchids threatened with extinction within their native ranges is one of the highest priorities on the research agenda at the M.M. Gryshko National Botanical Garden, National Academy of Sciences of Ukraine (Cherevchenko et al. 2007). Up to now, many native orchids species from South-East Asia and South America were propagated at the National Botanical Garden through asymbiotic seed germination and tissue culture procedures (Cherevchenko, Kushnir 1986; Buyun et al. 2004; Cherevchenko et al. 2007).

Angraecum eburneum subsp. *superbum* (Thouars) H.Perrier was selected as one of the priority species for in-depth studies aimed at preserving propagated plants under artificial conditions in glasshouses within the temperate climate zone in order to protect this remarkable species from extinction outside its native range.

The *Angraecum* Bory species are famous for their spectacular white, nectariferous, long/short-spurred flowers and unique relationships with pollinators. At present, Angraecoid orchids are extensively investigated due to their highly specialized bird and hawk-moth pollination syndromes (Wasserthal 1997; Nilsson 1998; Micheneau et al. 2006) and endangered conservation status (Cribb et al. 2005, 2011; Whithman et al. 2011). However, despite interesting biology and threats to these species in the wild, there are very few reports on reproductive biology

(flowering phenology, pollination efficiency, reproductive success) of these fascinating orchids under glasshouse conditions (Cherevchenko, Kushnir 1986; Buyun 2011).

According to the most recent data on molecular phylogenetics of *Vandeae* (Carlsward et al. 2006; Micheneau et al. 2008), the genus *Angraecum* belongs to subtribe *Angraecinae sensu lato* (tribe *Vandeae*, subfamily Epidendroideae).

The *Angraecum* species occupy a wide range of habitats. *Angraecum eburneum*, a widespread species known to occur in both mainland Africa and Madagascar, may have both epiphytic and terrestrial growth habit (Cribb et al. 2011). *A. eburneum* is one of the species rather frequent in coastal forests. *Angraecum eburneum* subsp. *superbum* is native to Madagascar, Comoros and Seychelles (Pridgeon et al. 2014).

We consider studying the reproductive biology of rare tropical orchids as an important background for any conservational project *ex situ*. Therefore, the main objectives of this investigation were: (1) to study the flowering phenology of *A. eburneum* subsp. *superbum* plants; (2) to elucidate the influence of breeding systems on fruit set and seed viability; (3) to explore the metric parameters of flowers against their location within the inflorescence; (4) to determine the effect of flower position in the inflorescence on seed / fruit masses, as well as on seed morphometry and viability.

Materials and methods

Plant material

The research was conducted on plants of *Angraecum eburneum* subsp. *superbum* cultivated under glasshouse conditions at the M.M. Gryshko National Botanic Garden, NAS of Ukraine. Uniform ten to fifteen year-old plants were randomly chosen for flowering phenology observations and experimental pollination treatments.

Breeding system and seed viability assessment

To assess the effect of breeding system on fruit set and seeds viability, the following pollination treatments were performed: artificial self-pollination (induced autogamy) and artificial cross-pollination (induced xenogamy). Self-pollinations were carried out by hand using forceps, pollinating flowers with their own two pollinia. Forty flowers on eight inflorescences from eight individuals were pollinated (five flowers per inflorescence). Cross-pollinations were performed by hand, pollinating flowers by the pollinarium with two pollinia from distinct plants. As in self-pollination treatment, forty flowers on eight inflorescences from eight individuals were pollinated (five flowers per inflorescence). We did not undertake an autogamy experiment (enclosing plants in nylon bags to exclude pollinia transfer), because during many years of observation we found no evidence of autogamous self-pollinations in this orchid. Fruit initiation and development was monitored until fruits were mature (about six months).

To assess the degree of self-incompatibility, a self-incompatibility index (ISI) was used (Ruiz-Zapata, Arroyo 1978).

To determine the viability of seeds resulted from induced autogamy versus artificial xenogamy, capsules from hand self- and cross-pollinations were harvested just before the capsules split open. For assessing seed viability, ten samples of 100 seeds (each sample from ten distinct fruits) for both pollination treatments were scored for viability on a morphological basis only: seeds with well-developed embryos were considered viable, whereas seeds with no embryo or a rudimentary embryo were considered inviable (Borba et al. 2001).

Effect of flower position on reproduction success

To estimate association between morphometric parameters of various flower parts (sepal, petals, labellum, column, and spur) of the species studied and their position in inflorescence, linear regression analysis was conducted based on the methodological approach of Bateman and Rudal (2000). Six distinct plants with inflorescences bearing eight flowers were randomly chosen. The measurements were made at the peak of flowering when all flowers within inflorescences were open.

Comparison of reproductive potential of flowers in different positions were made using inflorescences (n = 6)

with equal flower number (at least eight flowers or more) from six individuals of *A. eburneum* subsp. *superbum* to ensure that the data obtained are comparable. In order to reduce the influence of pollen from the flowers of other individuals, all eight flowers within the same inflorescences were pollinated by their own pollinia (induced autogamy). The flowers were hand pollinated in a few days after they opened.

Seed morphometry and viability

The average length and width of seed coat and embryo were determined, as well as the percentage of seeds with embryos. The volumes of seeds and embryos were defined with the formulas (Arditti, Ghani 2000):

$$V_S = 2 \times \left[\left(\frac{w}{2} \right)^2 \times \frac{l}{2} \times \frac{\pi}{3} \right],$$

where V_S – volume of seed; w – width of seed; l – length of seed;

$$V_E = \frac{4}{3} \pi \times \frac{l}{2} \times \left(\frac{w}{2} \right)^2,$$

where V_E – volume of embryo; w – width of embryo, l – length of embryo;

$$A_S = \left(\frac{V_S - V_E}{V_S} \right) \times 100\%,$$

where A_S – volume of air space in a seed.

In the study 100 seeds of every sample were used.

Fresh flowers were dissected to examine the general morphology of gynostemium and pollinia. All measurements of perianth parts and fruits dimensions were taken using digital caliper and ruler. Weighing of fruits and seeds was carried out with a Kern ABS/ABJ 320-4M analytical balance (Kern & Sohn GmbH, Germany).

The flower structures were photographed with a stereoscopic microscope Stemi 2000 C (Carl Zeiss, Jena, Germany). For the morphometric seeds/embryos research, a light microscope Primo Star B 48-0071 (Carl Zeiss, Jena, Germany), equipped with digital camera Canon PowerShot A640, was used. Image analysis and measurements were taken using AxioVision SoftwareTM Rel. 4.7 (Carl Zeiss, Jena, Germany).

Data were analyzed by Student's t-test and differences were considered statistically significant when $P \leq 0.05$. Statistical analyses were performed using Microsoft Office Excel 2007 as well as Statistica Software Package v. 8.0. The graphs were plotted with Microsoft Office Excel.

Results and discussion

A. eburneum subsp. *superbum* is an epiphytic orchid with monopodial growth habit. Robust stems of these plants are erect, with numerous strap-like leaves, distichously arranged, fleshy and unequally bilobed at the tip.
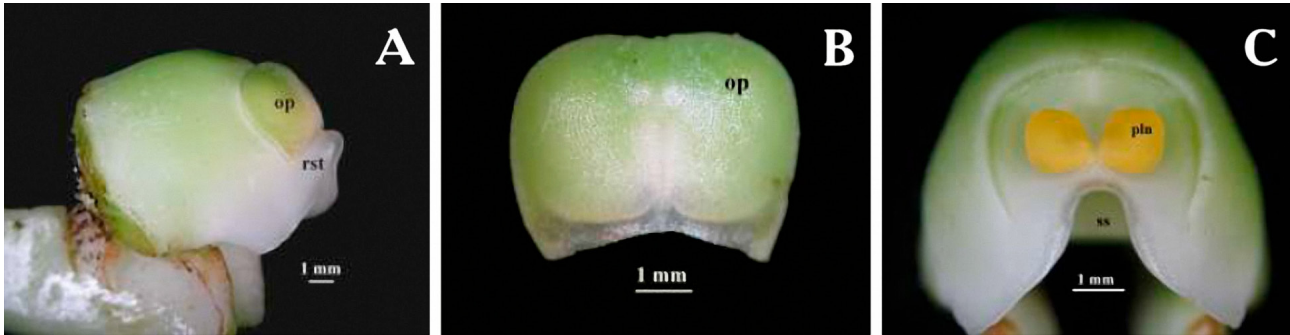
Fig. 1. Reproductive structures of *Angraecum eburneum* subsp. *superbum* flower. A, gynostemium, side view; B, operculum; C, gynostemium with pollinia attached. op, operculum; rst, rostellum; pln, pollinia; ss, stigmatic surface.

Inflorescences are axillary, many-flowered, 50 to 60 cm long. Plants usually produce one to four erect racemes of six to fourteen resupinate flowers, flesh in texture, with medium diameter 8.5 to 9.0 cm, alternately arranged on the inflorescence axis. Sepal and petal are free and subsimilar. Labellum is entire, with spur, projected backward. The spur is conical in shape, 5.5 to 6.0 cm long. As demonstrated in Fig. 1A & B, the column is massive, lacking a foot, with two pollinia. The rostellum is deeply cleft, while the stigma is deeply concave, elliptic (Fig. 1C).

The flowering period of the plants under glasshouse conditions extended from the end of November to the middle of February. The buds of *A. eburneum* subsp. *superbum* opened in late afternoon. Flowering peak occurred mainly in mid-January, with up to ten to twelve open flowers per inflorescence being observed. *A. eburneum* subsp. *superbum* has long-lasting flowers, with the perianth remaining fresh for about 1 to 1.5 months unless it is pollinated.

Although many studies have shown that pollination decreases floral longevity in orchids (Clayton, Aizen 1996; Luyt, Johnson 2001; Stpiczynska 2003; Abdala-Roberts et al. 2007; Buyun 2011; Huda, Wilcock 2012), we did not observed a rapid senescence of pollinated flowers of *A. eburneum* subsp. *superbum*. Approximately in five to seven days after pollination, the labellum was rolled up, although the other parts of perianth did not exhibit any changes for about one week after pollination (Fig. 2). Such pollination response of *A. eburneum* subsp. *superbum* can be explained by costs invested to tall flowering plants. These data confirm the relationship between reproductive costs (investment to flower construction and maintenance) and floral longevity, which has been previously shown for some other orchids, such as *Trichocentrum ascendens* (Lindl.) M.W. Chase & N.H.Williams [syn. *Cohniella ascendens* (Lindl.) Christenson] (Abdala-Roberts et al. 2007) and *Rhyncholaelia glauca* Lindl. (Schltr.) (Flores-Palacios, García-Franko 2003). After pollination the flowers of *A. eburneum* subsp. *superbum* still remained rather attractive, thus reproductive success of the plants increased.

Unlike many other orchid species with the most long-lasting flowers in lower position, in *A. eburneum* subsp. *superbum* the lowest-positioned flowers had shorter longevity than those in the middle and apical parts of the infloresence. This can apparently be attributed to the pollination syndrome of this sphyngophilous species.

Intact flowers remained fresh and receptive over one month. However, capsules produced from flowers pollinated at the end of life span (in a month time or more after flowering started) were smaller and lighter than those developed after pollination of a freshly opened flower. Moreover, apart from more than twice reduced fruit set, the delayed flower pollination resulted in considerably increased percentage of seeds without embryos or with rudimentary embryos.

In our observations, the duration of anthesis of a single inflorescence did not exceed nine weeks. The longevity of fruit maturation was about 5 to 5.5 months (Fig. 3).

The results of experimental pollination treatments (induced autogamy/artificial cross-pollination) are presented in the Table 1. The fruit set after self- and cross-pollination was significantly different (73 versus 95%), which is consistent with literature data (Micheneau et
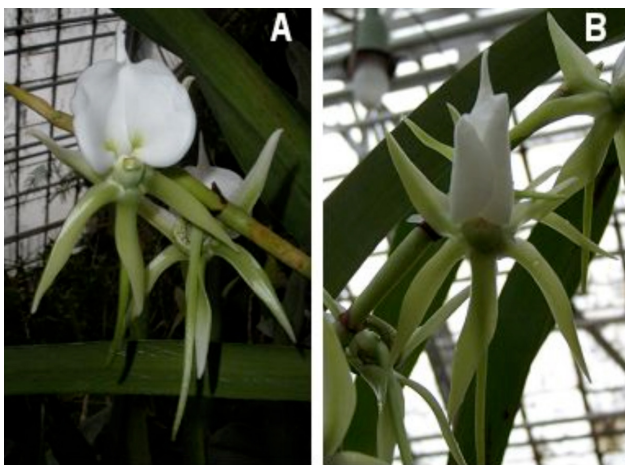

Fig. 2. Response of *Angraecum eburneum* subsp. *superbum* flowers to pollination. A, intact flower before pollination; B, pollinated flower one week after pollination (labellum was rolled up).

Fig. 3. Dehiscent fruit of *A. eburneum* subsp. *superbum* after full maturation.

al. 2006). In addition, the mean percentage of seeds with viable embryos per fruit per plant was higher in the cross-pollination treatment (97 % versus 65 %, respectively). In order to assess the degree of self-incompatibility, the index of self-incompatibility (ISI) (Ruiz-Zapata, Arroyo 1978) was determined as the proportion of fruits produced after self-pollination to fruits initiated from cross-pollination (29:38). The ISI ranges between zero (self-incompatibility) and one (self-compatibility); therefore the obtained value (ISI = 0.76) indicates that *A. eburneum* is self-compatible, though it is also possible to obtain values higher than one in the case when fruit set initiated by cross-pollination is lower than that obtained after self-pollination (Ruiz-Zapata, Arroyo 1978).

Variation in reproductive success between self- and cross-pollinations has indicated that despite self-compatibility, *A. eburneum* subsp. *superbum* possessed some degree of incompatibility or inbreeding depression under glasshouse conditions.

Furthemore, in this study we observed a unique flower opening pattern, rather unusual for orchids with many-flowered inflorescences: the fourth flower numbered from the inflorescence base was the first to start opening. After that, the following flowers within inflorescence opened sequentially, in both basipetal and acropetal successions. Therefore we regard *A. eburneum* subsp. *superbum* to be an appropriate model to study intra-inflorescence variations in flower dimensions and position-dependent reproductive success of flowers, relationship between reproductive costs (e.g. flower construction, maintenance), and floral longevity.

Unfortunately, we have not found in the literature any

data on natural fruit set for *A. eburneum* subsp. *superbum*, although it is widely known that fruit production in open-pollinated flowers for most species studied in nature is significantly lower than for hand-pollinated flowers (Berry, Calvo 1991; Borba et al. 2001; Flores-Palacios, García-Franko 2003).

For many angiosperm species, including the orchids, it has been shown (Berry, Calvo 1991; Medrano et al. 2000; Vallius 2000) that flowers at various position within an inflorescence possess differential reproductive success. To explain this phenomenon, a few hypotheses have been proposed. The "resource limitation" hypothesis (Vallius 2000; Abdala-Roberts et al. 2007) or "architectural effect" impact (Diggle 1995) proposed that the lowermost flowers in inflorescences or flowers opening first, displaying the highest probability of fruit initiation and maturation. At the same time, flowers located in distal positions within an inflorescence, have both smaller dimensions and lower reproductive potencies. This decrease may be contributed to competition among developing fruits for resources (Diggle 1995; Vallius 2000) or morphological discrepancy between the orchid flower and pollinator dimensions.

Therefore, to explore whether perianth traits within an inflorescence tend to decrease, due to so called "architectural effect", observed by other authors (Berry, Calvo 1991; Diggle 1995; Vallius 2000) in some orchids, we measured eight morphometric parameters (labellum length/width, petal length/width; column hight/diameter, spur length/diameter) for flowers in different positions.

In Fig. 4 A, B the graphs of flower metric parameters regressions against their positions within inflorescences are provided. A moderate increase of morphometric parameters (from the first to the third flower) occurred followed by their subsequent reduction. Afterwards, the regressions reveal weak parallel declines in labellum width and length from the base to the apex of the inflorescences. In contrast, spur width, column height and width, were constant throughout the inflorescence. Thus, we observed no clear manifestation of the so called "architectural effect" (Diggle 1995) within inflorescences of *A. eburneum* subsp. *superbum*.

In addition, there were no differences in the probability of pollination between flowers in different positions: all eight pollinated flowers initiated fruits. These results suggested absence of any functional restrictions in the flowers occupying different positions on inflorescences.

The position of pollinated flowers within the inflorescence had impact upon the mass of fruits and

Table 1. Comparison of fruit-set rates and seed viability of *A. eburneum* subsp. *superbum* under different experimental treatments (mean ± SE). Means followed by the different letters in each column are significantly different at $P < 0.05$

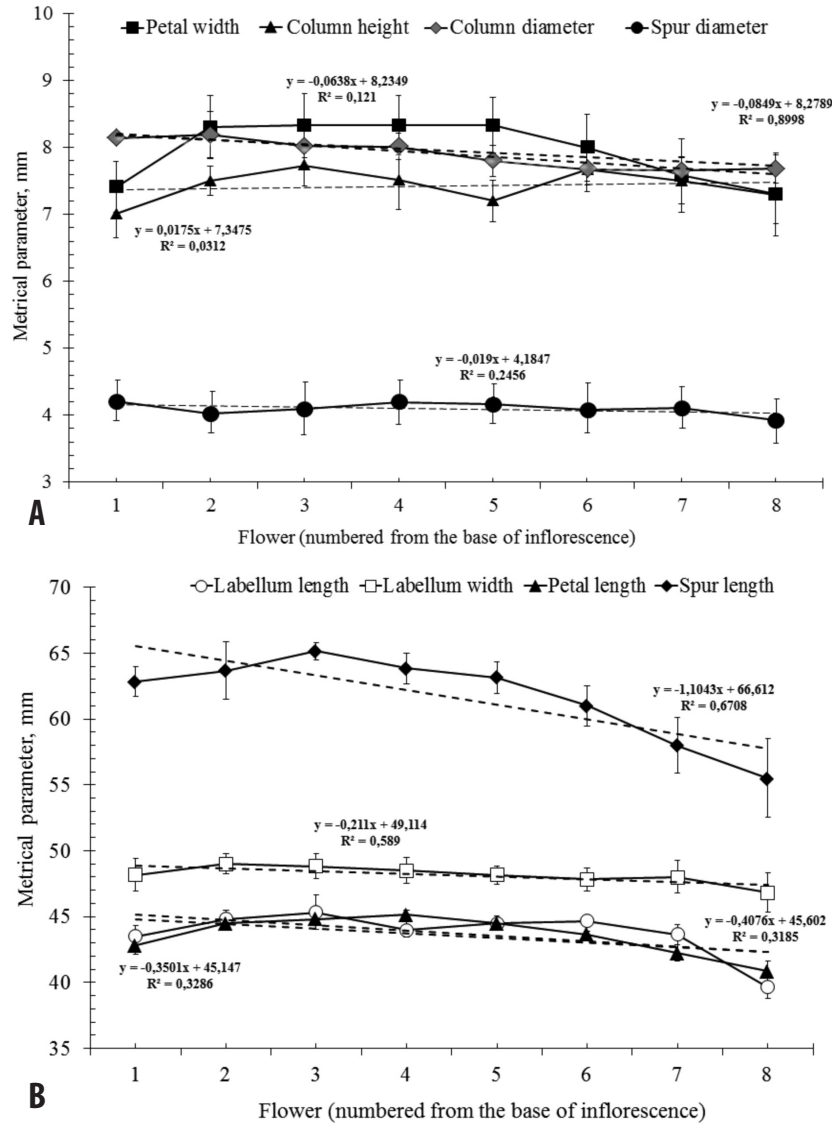| Treatments	Number of pollinated flowers	Number of ripen fruits	Fruit-set (%)	Seed viability (%)
Induced autogamy (self-pollination)	40	29	72.60 ± 2.28[a]	65.14 ± 1.94[a]
Artificial xenogamy (cross-pollination)	40	38	95.00 ± 3.16[b]	97.23 ± 3.87[b]

Fig. 4. A, regressions of the four metric parameters (petal width, column height/diameter, spur diameter) against location of the flower along inflorescence of *A. eburneum* subsp. *superbum*. Error bars represent standard errors. Dashed lines represent general trends in observed data as described by the linear regression equations. B, regressions of the four metric parameters (labellum length/width, petal length, spur length) against location of the flower along inflorescence of *A. eburneum* subsp. *superbum*. Error bars represent standard errors. Dashed lines represent general trends in observed data as described by the linear regression equations.

seeds. The weight of capsules decreased from the bottom to the top of inflorescence, whereas seed viability was not significantly different at various positions (Table 2).

The fruits formed in the lowest position in the inflorescence demonstrated both the largest fruit and seed masses and the highest percentage of seeds with developed embryos. Therefore, we conclude that reproductive success of *A. eburneum* subsp. *superbum* under glasshouse conditions is resource limited.

Our results are consistent with those of earlier research, which showed differential fruit set by flowers at various positions on inflorescences for several orchid species (Berry, Calvo 1991; Vallius 2000). In all of these cases, fruit set increased toward the bottom of the inflorescence and

decreased steadily toward the top.

As shown in Table 2, the decrease in capsule weight was not associated with seed viability, since the percentage of embryos in the seeds was not affected by the position of pollinated flower.

The highest percentage of viable seeds was observed in both the lowermost and the uppermost fruits, resulting in some contradiction simultaneously both with "resource limitation" and "architectural effect" hypotheses.

Apparently, to explain some differences in seeds viability between fruits initiated at different positions on inflorescence, it is necessary to take into account not only influence of "architectural effect", but as well the age of flower before pollination. A life span of the flower is known

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Table 2. Morphometric characteristics of fruits and seed viability of *Angraecum eburneum* subsp. *superbum* depending on fruit location within the inflorescence (numbered from the base of inflorescence). Each value represents the mean ± SE. Means followed by the same letters in each column are not significantly different at *P* < 0.05

No.	Capsule length (mm)	Capsule width (mm)	Capsule mass (g)	Seed mass (g)	Seed viability (%)
1	73.11 ± 0.81[a]	20.76 ± 0.63[a]	1.94 ± 0.16[a]	0.79 ± 0.09[a]	76.62 ± 2.45[a]
2	70.94 ± 0.78[ab]	20.77 ± 0.68[a]	1.63 ± 0.13[a]	0.44 ± 0.01[b]	73.43 ± 2.21[a]
3	68.52 ± 0.76[bc]	19.92 ± 0.81[a]	1.62 ± 0.19[a]	0.43 ± 0.02b[c]	72.81 ± 2.16[a]
4	66.74 ± 0.74[cd]	21.01 ± 0.61[a]	1.35 ± 0.17[ab]	0.39 ± 0.05[bd]	72.49 ± 2.18[a]
5	65.66 ± 0.70[de]	21.11 ± 0.71[a]	1.43 ± 0.17[a]	0.40 ± 0.08[be]	74.85 ± 2.88[a]
6	64.52 ± 0.58[e]	19.25 ± 0.74[ab]	1.29 ± 0.15[ac]	0.33 ± 0.03[bf]	71.44 ± 2.39[a]
7	56.73 ± 0.43[f]	17.79 ± 0.69[b]	1.18 ± 0.14[ad]	0.42 ± 0.02[bg]	71.30 ± 2.78[a]

to correspond to its reproductive potential: the longer a period before pollination, the lower reproductive success of the flower.

The data on the morphometry of seeds and embryos are presented in Fig. 5. The seeds of the lowest-positioned fruit possessed both the largest seeds and embryos.

The obtained results are somewhat contradictory. While the lowermost flowers do not open first, they apparently have an advantage in competition for resources with previously opened flowers located in the middle of the inflorescence.

Conclusions

The results of pollination treatments indicated that *A. eburneum* subsp. *superbum* is self-compatible but not autogamous. We have observed that the first flower to start opening is located in the middle part of inflorescence, demonstrating a unique opening succession pattern for orchids. The main factors affecting the efficiency of reproduction of *A. eburneum* subsp. *superbum* under

glasshouse conditions are both breeding system and resource availability. The results demonstrate positive effect of cross-pollination both on fruit set and seed viability of *A. eburneum*, which is not uncommon for other orchids. Pollinated flowers of the studied species showed extended flower longevity after pollination, which confirms the relationship (trade-off) between investment to flower construction, its maintenance and floral longevity.

The present investigation contributes to the knowledge on the diversity of reproductive strategies in tropical orchids under artificial conditions, *ex situ*. In addition, the viable seeds obtained in our research will be used for propagation of *A. eburneum* subsp. *superbum* through *in vitro* culture for long-term conservation of these plants under glasshouse conditions.
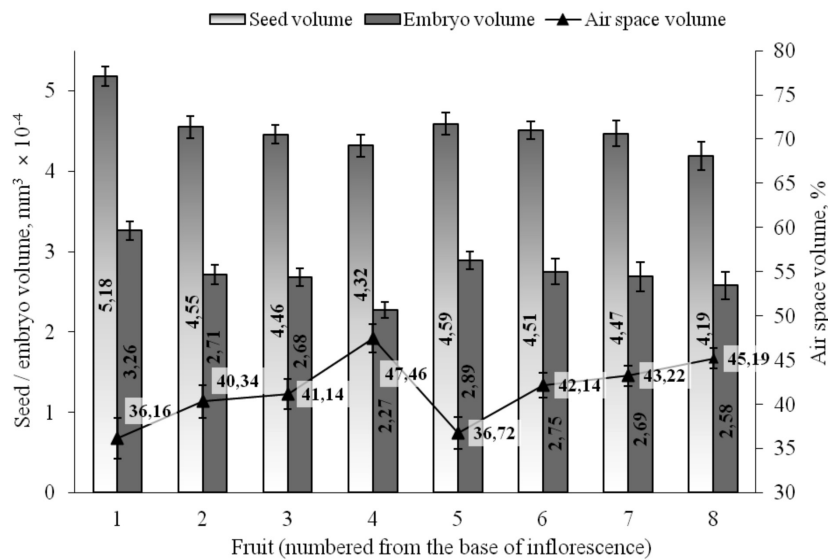
Fig. 5. Morphometry of seeds/embryos from fruits in different locations along the inflorescence of *Angraecum eburneum* subsp. *superbum*. Bars represent standard errors.

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