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Evaluation of the possibility of obtaining viable seeds from the cross-breeding *Hippeastrum* × *chmielii* Chm. with selected cultivars of *Hippeastrum hybridum* Hort.

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ABSTRACT

Hippeastrum sp. is a popular cut flower and a popular potted plant, currently occupying the 11th position among cut flowers sold on the Dutch flower auctions. *Hippeastrum* × *chmielii* was bred by Henryk Chmiel at the Warsaw University of Life Sciences (WULS), Poland. The aim of this study was designed to test the ability of two clones of *H.* × *chmielii* to reproduce generatively by pollinating by three cultivars of *Hippeastrum hybridum* – 'Gervase', 'Rio Negro' and 'Royal Velvet'. Pollen viability was tested by germination on the medium and by acetocarmine staining. The receptiveness of the stigmas and ovules of *H.* × *chmielii* and thus their ability to possibly accept compatible pollen were checked by staining with red alizarin. The viability of pollen grains of tested cultivars was estimated at 66.4–83.0% and their high ability to develop pollen tube was found. It was determined that the embryos of both *H.* × *chmielii* clones were fully receptive. A total of 72 crossings were performed in 6 combinations. Seeds collected about 1 month after pollination were germinated immediately after harvest either on moist tissue paper on Petri dishes or in jars with distilled water. After 28 days the percentage of germinated seeds ranged between 48.3% and 77.9%, for different crosses. In the case of seeds obtained from crossing *H.* × *chmielii* clone 18 × *H. hybridum* 'Gervase' a higher average percentage of germinated seeds was obtained in jars while no differences were noted between the germination methods in other cases.

Keywords: amaryllis, fertilisation, germination, pollen, pollen tube, stigma

INTRODUCTION

Hippeastrum sp. (commonly known as amaryllis in the global market) currently occupies the 11th position among cut flowers and foliage sold on the Dutch flower auctions (CBI Market Intelligence, 2016) and it's also a popular potted plant worldwide. The genus *Hippeastrum* is still not very well understood taxonomically (Tombolato et al., 2013), particularly that it was included in the genus *Amaryllis* till 14th International Botanical Congress in 1987. *Hippeastrum* is a native bulbous plant from America (Bryan, 2002), including 55–75 species distributed from Mexico to Argentina (Tombolato et al., 2013). Wild species – mainly from Brazil and Peru – gave origin to new hybrids and hundreds of them are now cultivated worldwide as *Hippeastrum hybridum* Hort. cultivars. According to Traub (1958), *Hippeastrum* obtained from the crossing of several selected species, specifically *Hippeastrum vittatum* with *H. aulicum*, *H. reginae*, *H. leopoldii* and *H. pardinum*. Breeding began with *Hippeastrum* × *johnsonii* (*Amaryllis* × *johnsonii* at that time), a species commonly regarded as the first hybrid form obtained in England in 1799, which was created by the crossing of *H. vittatum* (*A. vittatum*) with *H. reginae* (*A. reginae*) (Meerow, 2009; Okubo, 1993; van Dijk and Kurpershoek, 2002).

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Then, for almost 200 years, the breeders had been searching for ideal forms and created new cultivars and new hybrid species and among them was the *Hippeastrum* × *chmielii* Chm. bred by Professor Henryk Chmiel in 1993 at the Department of Ornamental Plants, Warsaw University of Life Sciences (WULS) – SGGW, Poland. The selected clones of the inter-species hybrid (*H. pratense* (Poepp.) Baker (= *Rhodophiala pratensis* (Poepp.) Traub) × chosen cultivars of *H. hybridum*) were characterised by vigorous growth (formation of large green mass), no visible resting period and repeated flowering. However, the periods of late winter and early spring were observed as the most abundant flowering periods for this species (Chmiel, 2000; Chmiel and Ilczuk, 2002; Chmiel et al., 2002; Ilczuk, 2015). The flowers were characterised by new decorative values. They were smaller than those of the compact hybrids, which may give the reason for their classification under the Diamond Group or Colibri Group (Van Scheepen et al., 2007). The delicate and small flowers made the material easier to pack and transport. In floristry, the petite structure has also overcome the difficulty of arranging the large, massive and visually quite heavy flowers of the standard cultivars of *H. hybridum*.

Depending on the clone, the petals can be red, red orange, brick orange, or bicolour with a star-shaped throat. Inflorescences remain fully decorative for about 13 days (Chmiel and Mynett, 1997; Chmiel and Szymański, 2001; Chmiel et al., 2002).

According to the register reports of the Dutch Royal General Bulb Growers' Association (KAVB), the biggest number of cultivars were registered for three groups with single flowers – Galaxy, Diamond and Colibri during the years 2016–2019. The reason for this may be the constantly growing demand for this type of flower (Bodegom and van Scheepen, 2017, 2018, 2019; Bodegom et al., 2020).

The bulbs of the *H.* × *chmielii* have a smaller circumference and are more flattened than those of the compact cultivars of *H. hybridum*, which makes it possible to plant more bulbs per square meter. Production for cut flowers is therefore more profitable (Chmiel and Ilczuk, 2002), as more flowers can be obtained from a smaller growing area. A high coefficient of vegetative propagation was observed for all clones of *H.* × *chmielii*. Depending on the circumference of the storage organ, the plants produced a large number of daughter bulbs, which make this species propagate easily (Chmiel and Mynett, 1997; Chmiel et al., 1998; Chmiel and Szymański, 2001; Chmiel et al., 2002; Ilczuk, 2005).

No one had so far tried to propagate *H.* × *chmielii* by seeds as well as checked the possibility of using the clones for further breeding. This study aimed to test the ability of two *H.* × *chmielii* clones – No. 6 and No. 18 to reproduce generatively by pollinating by three cultivars of *H. hybridum* – 'Gervase', 'Rio Negro' and 'Royal Velvet'.

MATERIALS AND METHODS

Plant material

The research was conducted on *Hippeastrum* × *chmielii* Chm. clones No. 6 and No. 18 (Figure 1) and three cultivars of *H. hybridum* – 'Gervase', 'Rio Negro' and 'Royal Velvet' (Figure 2). The cultivars 'Gervase' and 'Royal Velvet' are of Dutch origin and they belong to the Galaxy group. The diameter of their flowers is >16 cm. The first cultivar has pink-red flowers while the second one is a dark burgundy. 'Rio Negro' is a cultivar from the Spider group whose flowers are similar to the shape of a spider. It also has an interesting flower colour and anthocyanin pigmentation of the inflorescence stem. Two clones of *H.* × *chmielii* Chm. were selected for the study. Clone No. 6 has a dark red colour, and clone No. 18 has a brick orange colour. The first one was typical for the group of red coloured clones, and the second one was typical for the group of orange coloured clones. Both clones have flowers with a diameter of 12–16 cm and are characterised by the production of a large number of daughter bulbs.

The bulbs of *H.* × *chmielii* Chm. were collected from the Department of Ornamental Plants of Warsaw University of Life Sciences – SGGW, Poland (WULS), where this hybrid was bred by Henryk Chmiel. The bulbs of cultivars originated from the commercial stocks.

The bulbs were cultivated in the greenhouse in plastic boxes 60 × 40 × 22 cm in a medium consisting of peat substrate and perlite at 3:1, supplemented with 10 g per box of complete fertilizer for bulbous plants (Agrecol Sp. z o.o., Poland) (7% N, 24% K_2O, 13% CaO) and adjusted to pH = 6.5. The plants were grown from the beginning of April to the end of June 2018 at 24–30°C (on some hot days the temperature rose to 32°C) at natural photoperiod (day length of 13–17 h) without light supplementation. All experiments were conducted from April to July 2018 at WULS.

Preparing flowers for pollination

First, stamens were mechanically removed by laboratory tweezers from the fully coloured but unopened flower buds for preparing the flowers of *H.* × *chmielii* for pollination. The stigmas were protected by aluminium foil against uncontrolled or self-pollination.

Evaluation of pollination and fertilisation ability of flowers

The pollination ability of flowers of *H.* × *chmielii* clones was checked by staining the stigmas and ovules with alizarin red on the microscopic slides for 5 min. Three repetitions of microscopic slides for two flowers of each clone were prepared. The solution was prepared according to McGee-Russel (1958) by dissolving 2% alizarin red (Sigma Aldrich) in a solution of ammonia adjusted to pH = 4.2. In the case of *Hippeastrum* cultivars, indirect pollen viability was assessed by staining with 1% acetocarmine (10 g of carmine dye dissolved in 1 L of 45% glacial acetic acid). From the



Figure 1. Clones of *H.* × *chmielii* – No. 6 (left) and No. 18 (right).



Figure 2. Cultivars of *H. hybridum* tested – 'Gervase', 'Rio Negro' and 'Royal Velvet' (from left to right).

prepared microscopic slides and after 5–10 min of staining, the number of stained and all visible pollen grains were counted under an AX Provis (Olympus) light microscope (Olympus Optical Co. Ltd., Japan) at a magnification of 100×. Properly developed viable pollen grains were coloured carmine while the non-viable ones remain non-stained. The percentage of visible pollen grains for each cultivar was calculated based on their numbers observed in three fields of view. The germination capacity of pollen grains was evaluated by germination of 150 grains (in three repetitions) on the 50 mL agar solidified medium on a Petri dish lined with filter paper soaked with distilled water, containing 10% sucrose, 100 ppm H_3BO_3, 300 ppm Ca $(NO_3)_2 \cdot 4H_2O$, 200 ppm $MgSO_4 \cdot 7H_2O$ and 100 ppm KNO_3 (Brewbaker and Kwack, 1963). The ability of pollen grains to develop pollen tubes was observed after 24 h under an AX Provis (Olympus) light microscope at a magnification of 100×.

Pollination and fertilisation

In May 2018, selected flowers of both clones of *H.* × *chmielii* were cross-pollinated by pollens taken from three cultivars. The following crosses were made: *H.* × *chmielii* clone 18 ♀ × *H. hybridum* 'Gervase' ♂ (10 crosses), *H.* × *chmielii* clone 18 ♀ × *H. hybridum* 'Royal Velvet' ♂ (15 crosses), *H.* × *chmielii* clone 18 ♀ × *H. hybridum* 'Rio Negro' ♂ (17 crosses), *H.* × *chmielii* clone 6 ♀ × *H. hybridum* 'Gervase' ♂ (10 crosses), *H.* × *chmielii* clone 6 ♀ × *H. hybridum* 'Royal Velvet' ♂ (15 crosses), *H.* × *chmielii* clone 6 ♀ × *H. hybridum* 'Rio Negro' ♂ (10 crosses).

Further, callose staining was performed 24 h after pollination to determine the ability of the pollen tube in penetrating the ovary under a microscope. The collected test material was placed in 70% ethanol for 24 h, next macerated for 1.5 h in 10M NaOH in an incubator (40°C) and followed by three washes in distilled water for 20 min. Then, the material was dyed for 1 h in 0.05%

aniline blue solution in 0.067M phosphate buffer in total darkness. The microscopic slides (in three repetitions for each cross combination) were prepared with 50% glycerol, the fluorescence of callose was observed in AX Provis (Olympus) microscope (Olympus Optical Co. Ltd., Japan) using NU filters (excitation filter BP360-370 nm, barrier filter BA420 nm, dichroic mirror DM400 nm) and then photos were taken using Olympus U-CMAD 3, Japan, by QuickPHOTO Pro.

One week after pollination, mesh insulators were applied to the expanding ovaries to prevent them from cracking uncontrolled and the seeds from falling to the ground.

Seed germination

In mid-June 2018, entire seed bags were collected. The seeds from each bag (from 10 pcs. to 17 pcs. depending on crossing) were counted and divided into two parts for two germination methods testing. The first one is covered in Petri dishes which were lined with filter paper soaked in distilled water (ISTA 2011) and the second one in open glass jars of 240 mL volume were filled with 100 mL of distilled water (according to Treder – personal communication). The germination was taken place in a cultivation room at 21°C with 16 h of cold white fluorescent light at 25 µM · m^{-2} · s^{-1}, and 60% RH all the time. The conditions were the same for each seed germinating method. The number and percentage of germinated seeds were assessed after 28 days (according to ISTA 2011).

Results of pollen viability and seed germinating were analysed on transformed data using Bliss transformation by analysis of variance and Multiple Range Test at the 5% significance level, using Statgraphics Plus 4.1.

RESULTS

Evaluation of pollination and fertilisation ability of flowers

The pollination capacity of the flowers of *H.* × *chmielii* clones No. 6 and No. 18 was determined by staining the stigmas and ovules with alizarine red. The red colouring in all the prepared microscopic slides indicates the accumulation of Ca^{2+} ions in large quantities, which expresses their receptivity (Figures 3A,3B and 4A,4B). Pollen grain viability of *H. hybridum* cultivars assessed by acetocarmine staining method was found and it was at the level of 83.0% for 'Gervase', for 'Royal Velvet' it is 82.7% and for 'Rio Negro' it is 66.4% (Table 1) (Figure 5A–5C). Based on the microscopic observations, the pollen grains of all the examined cultivars, placed on an agar solidified medium, were characterised by their capacity to produce pollen tubes (Figure 6A,6B).

Pollination and fertilisation

Microscopic observations under a fluorescence microscope made 24 h after the application of pollen grains of three tested cultivars to the stigmas of both *H.* × *chmielii* clones confirm the presence of pollen tubes on fragments of the stigmas (Figure 7).

Seed germination

Pollinated flowers formed a different number of seeds. A detailed summary of the number of seeds obtained from individual crossings is shown in Table 2. From one flower a minimum of 22 and a maximum of 94 seeds were collected, on average for the type of cross-breeding from 44 seeds to 71 seeds. The least number of seeds was collected from the flowers of *H.* × *chmielii* clone No. 18 × 'Rio Negro' – 305, and the largest number from the flowers of *H.* × *chmielii* clone No. 6 pollinated with pollen from 'Royal Velvet' – 993. In the case of crossing of *H.* × *chmielii* clone No. 6 with *H. hybridum* 'Rio Negro', all seed bags became yellow and died on the inflorescence shoots and so no seeds were obtained. Totally, from all 72 crossings in 6 types, 3,043 seeds were obtained.

The first germinating seeds appeared after 9 days in jars with water. The percentage of germinated seeds calculated after 28 days varied between 48.3% and 77.9%, depending on the type of cross-breeding (Table 3). No effect of the germination method was noticed for most of the crossings. Only in the case of seeds originated

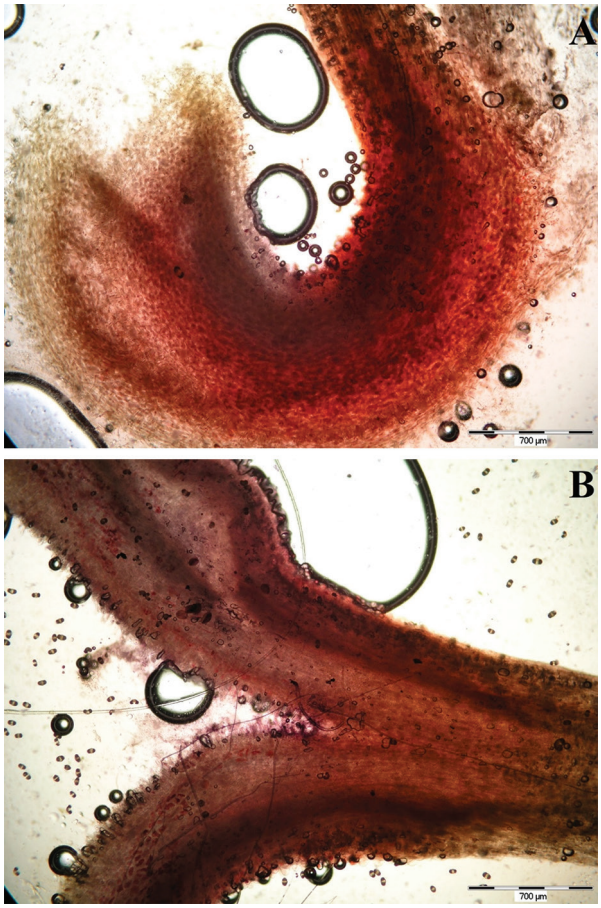


Figure 3. The receptive stigma of *Hippeastrum* × *chmielii* stained by alizarine red – clone No. 6 (A) and clone No. 18 (B).

from cross-breeding of *H.* × *chmielii* 18 × 'Gervase', more seeds germinated in jars with water than on Petri dishes.

DISCUSSION

Cross-pollination is the only method of obtaining seeds capable of germination in *H. hybridum* (Wóycicki, 1966; Almeida et al., 2019). Additionally, the flowers of *Hippeastrum* are proterandrous, which means that the anthers mature earlier than the stigmas (Almeida et al., 2019; Szlachetka, 2000). It confirms the necessity of artificial pollination. However, in the case of *H.* × *chmielii*, self-pollination can occur (Szlachetka, 2000). In practice, a common problem in the process of successful pollination and fertilisation is the occurrence of various types of cross-pollination barriers as reported by many authors (Bomblies and Weigel, 2007; Chen and Lin 2016; Kuligowska et al., 2015 – for Kalanchoe genus). In the present research, during the crossbreeding of the *H.* × *chmielii* clone No. 6 with *H. hybridum* 'Rio Negro', the seed bags died out, which may indicate the occurrence of prezygotic barriers.

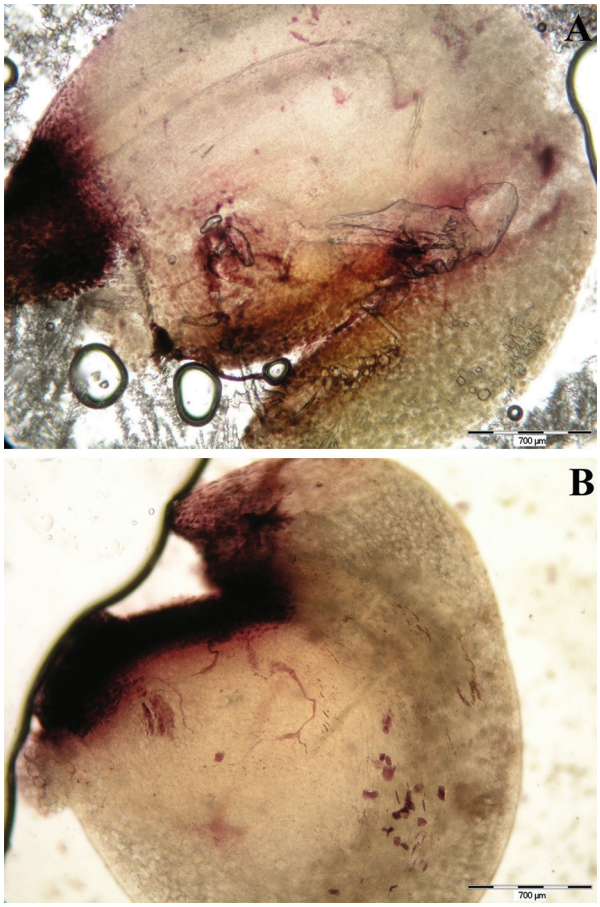


Figure 4. The receptive ovule of *Hippeastrum* × *chmielii* stained by alizarine red – clone No. 6 (A) and clone No. 18 (B).

In our study, high receptivity of the stigmas of both *H.* × *chmielii* clones was observed in alizarin red staining. Observed red colouring in all microscopic slides indicates the accumulation of Ca^{2+} ions in large quantities, which is related to the enzymatic decomposition of pectins (Brewbaker and Kwack, 1963; Reger et al., 1992). It can be confirmed that pectins are also present in the structures of the hippeastrum pistil, as described by Chudzik (2002) for other *Amaryllidaceae* species. The content of pectins is essential for overcoming the problems due to the distance from the stigma of the pistil to the ovule by pollen tubes. The distance from the stigma of the pistil to the ovules is different in different plants and ranges from a few millimetres in *Zantedeschia* to several centimetres in *Colchicum* (Wóycicki, 1966). The distance for the *Hippeastrum* was determined as ca. 15 cm by Traub (1958). The enzymes found in the pollen tube decompose pectins into free calcium ions, which are the chemo-attractants that direct the pollen tubes towards the receptive ovules (Malho et al., 2000). During the evaluation of the germination capacity of the pollen grains of tested cultivars on a medium that imitates the conditions of the stigma, the pollen tubes spread out in each direction without any visible reference point. According to Wóycicki (1966), the pollen tubes growing on artificial substrates are much longer than the pistils of some species. In the case of *Hippeastrum*, this was not confirmed. The absence of arabinogalactan proteins in the medium, which probably play a signalling role for pollen tube growth towards the mature ovary sac (Schultz et al., 1998; Śnieżko and Chudzik, 2003), was responsible for the lack of directed pollen tube growth under artificial conditions.

Table 1. Percentage of viable/stainable pollen grains of cultivars *H. hybridum* – 'Gervase'; 'Royal Velvet'; 'Rio Negro'.

Cultivars	Total number of pollen grain ± SD	Number of viable pollen grains ± SD	Percentage of viable pollen grains (%) ± SD
H. hybridum 'Gervase'	195 ± 4.08	162 ± 0.82	83.0 ± 0.06 b*
H. hybridum 'Royal Velvet'	177 ± 1.63	145 ± 3.30	82.7 ± 5.51 b
H. hybridum 'Rio Negro'	285 ± 4.08	210 ± 1.63	66.4 ± 9.21 a

*Means ± standard deviation in a column followed by the same letter does not differ significantly at $p = 0.05$.

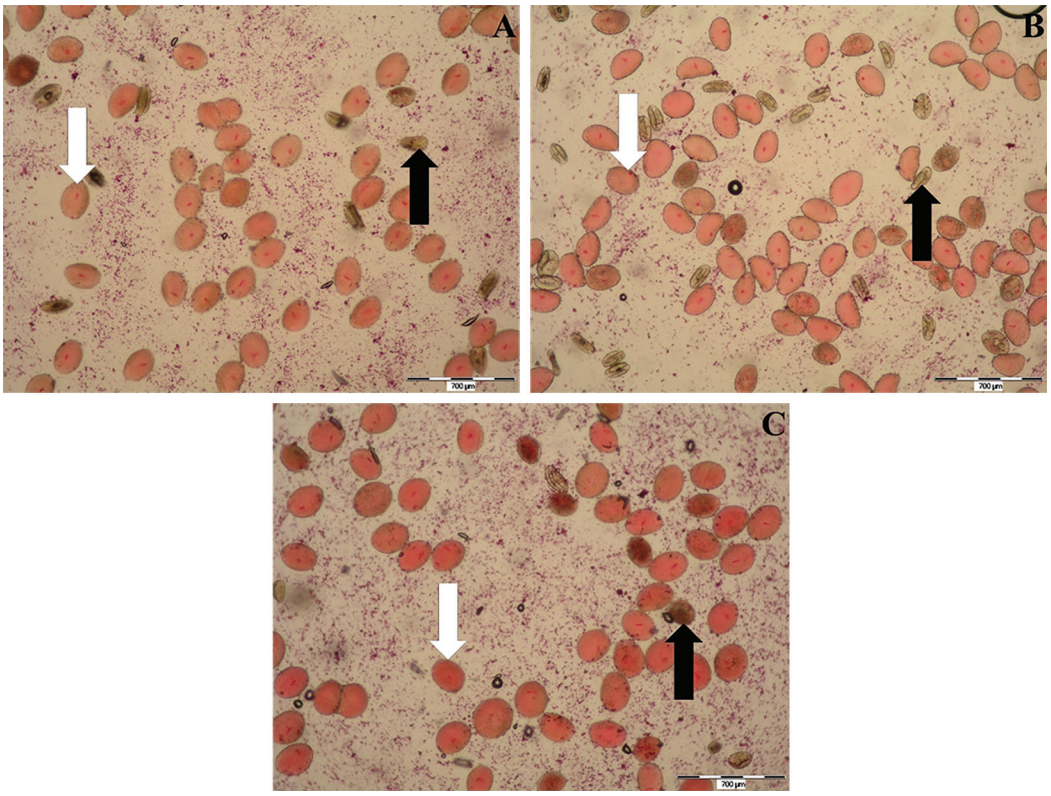


Figure 5. Pollen grains stained by acetocarmine – *H. hybridum* 'Gervase' (A), *H. hybridum* 'Rio Negro' (B) and *H. hybridum* 'Royal Velvet' (C). White arrows show viable pollen grains; black arrows show non-stainable/non-viable grains.

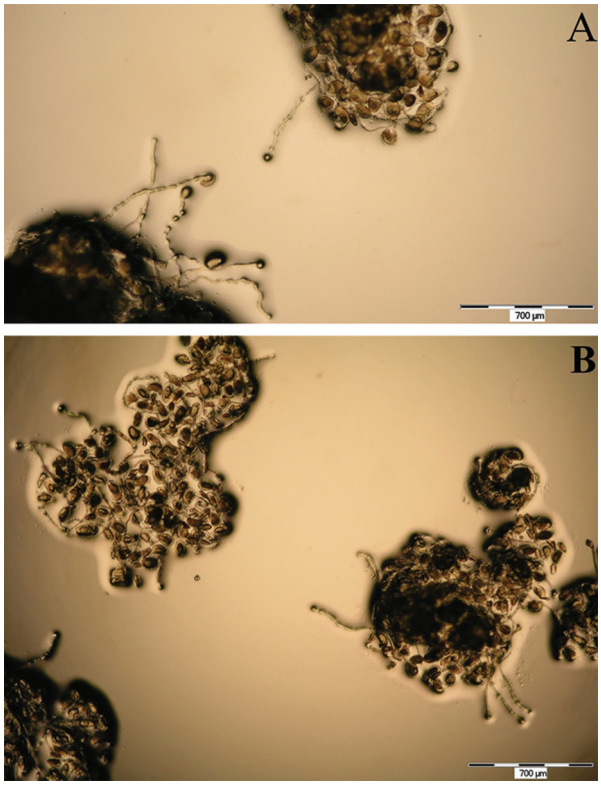


Figure 6. Sprouting pollen tubes on the germination medium – *H. hybridum* 'Rio Negro' (A) and *H. hybridum* 'Royal Velvet' (B).

In our study, the viability of *Hippeastrum* pollen grains was evaluated after the opening of the anthers by acetocarmine staining. Similar methods were used in the studies conducted by Weryszko-Chmielewska and Chwil (2006) for *Taraxacum officinale*, Szklanowska (1992) for selected trees and shrubs of the *Rosaceae* family and by Chwil (2006) for *Narcissus* sp., which were the most related to the *Hippeastrum*. On the other hand, it is known, that the viability/stainability of pollen grains is dependent on the method of staining (Słomka et al., 2010). In all cultivars of *H. hybridum* in our research, the pollen viability did not exceed 83%, but the lowest was 66.4%. In *Narcissus* (*Amaryllidaceae*), Chwil (2006) determined the pollen grains viability up to 92% in cultivar 'Hardy' and only 22% in cultivar 'The Sun'. The viability of pollen grains ranged from 62% to 77% (Chwil, 2006) for the remaining cultivars of *Narcissus* and was comparable to the level of viability observed for three *Hippeastrum* cultivars tested by us and at the level of 60–80% for nine *Hippeastrum* hybrids reported by Khaleel et al. (1991). Therefore, the pollen viability could be a cultivar feature. Many cultivars obtained by crossing show reduced pollen viability or even sterility. This was proven, among others, for nine lily genotypes in a study by He et al. (2017), where the percentage of germinating pollen 1 day after anthesis was 81% for *Lilium sulphureum,* 73.4–77.1% for three hybrid cultivars and only 17.8% for cultivar 'Tiny Padhye'. The pollen of

the cultivar 'Jinghe' did not germinate at all. For narcissus, Sanders (2014) reported that the number of germinated pollen grains from the samples collected was 400 for the cultivar 'Gloriosus' but only 20, 23 and 34 for 'Magic Step', 'Silver Bells' and 'Problem Child', respectively.

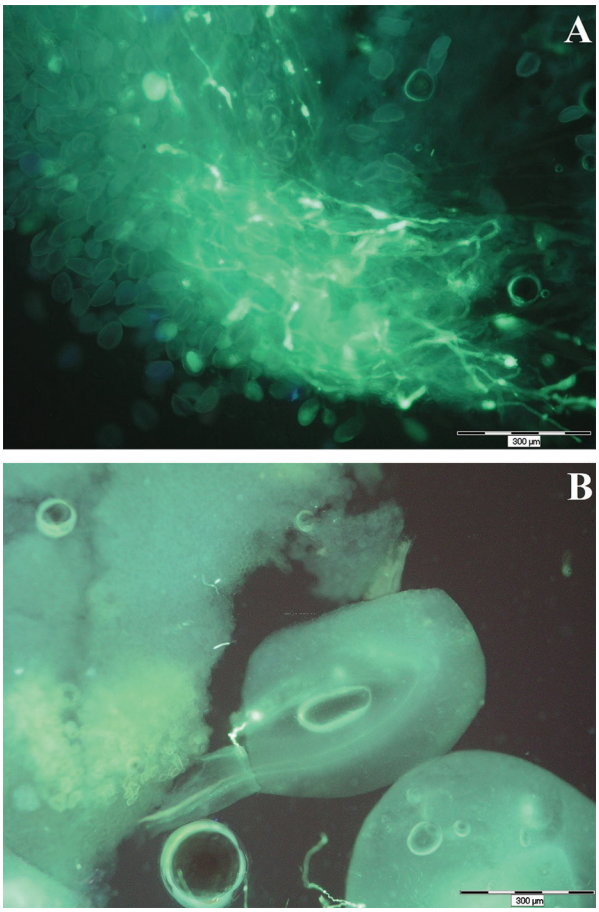


Figure 7. Germinating pollen grains of *H. hybridum* 'Rio Negro' on the stigma (A) and pollen tubes of 'Rio Negro' penetrated the ovule (B).

The seeds obtained in the experiment ripened for 3 weeks, after which the bags were cracked and the seeds started to fall down. A single seed bag contained a minimum of 22 seeds to a maximum of 94 seeds, and there is a literature report of seeds range of 40–80 in a single seed bag for *H. hybridum* (Kazimierczak, 1992; Okubo, 1993; Szlachetka, 2000). The first germinating seeds appeared after 9 days in jars with water, which meats ISTA standards (ISTA, 2011) of 7–10 days for germinating of *H. hybridum*. The percentage of germinated seeds after 28 days varied between 48.4% and 77.9% and was affected by the type of cross-breeding. Similar studies carried out by Arayakitcharoenchai (2012) on seeds, obtained from crossbreeding of hybrid forms, showed that the first seedlings appeared after 14 days. The germination capacity of seeds was evaluated after 30 days and the total percentage of germination was between 62.3% and 98.6%. The maximum viability of pollen in our trials did not exceed 78%. It could be negatively affected by the high temperature in the greenhouse during the flowering period of these plants. This is confirmed by Doijode (2001), which states that seed viability of *Hippeastrum* is lost completely within 3 months of storage at 25–35°C.

The above results concerning the propagation of *H.* × *chmielii* clone No. 6 and clone No. 18 by seeds as well as further research and selection work on the obtained seedling population may contribute to obtaining new, interesting cultivars of this species in the near future. An additional aspect can be the knowledge of physiological aspects of flower development, which is useful in practice and applied for breeding programs. This will help in the selection of forms to be crossed and in choosing the right time for pollination. The obtained results concerning seed germination are important for breeding and horticultural practices.

Table 2. The number of seeds obtained from individual crossings of *H.* × *chmielii* with three cultivars.

Crossing	Number of flowers	Number of collected seeds from one flower (min – max)	Average number of collected seeds per flower ± SD	Total number of seeds
H. × *chmielii* 18 × 'Gervase'	10	31–76	57.7 ± 15.53	577
H. × *chmielii* 18 × 'Royal Velvet'	15	24–86	59.1 ± 17.99	828
H. × *chmielii* 18 × 'Rio Negro'	17	22–68	43.6 ± 17.68	305
H. × *chmielii* 6 × 'Gervase'	10	25–94	48.6 ± 22.17	340
H. × *chmielii* 6 × 'Royal Velvet'	15	53–83	70.9 ± 9.44	993
H. × *chmielii* 6 × 'Rio Negro'	10	0	0	0
Total number of seeds from all crossings				3,043

Table 3. Percentage of germinated seeds of *H.* × *chmielii* pollinated by pollen of three cultivars depending on the germination method.

Crossing	Germination on Petri dishes (%)*	Germination in water in the jars (%)*
H. × *chmielii* 18 × 'Gervase'	55.0 ± 5.61 a**	71.2 ± 6.77 b
H. × *chmielii* 18 × 'Royal Velvet'	67.2 ± 9.54 a	70.6 ± 8.66 a
H. × *chmielii* 18 × 'Rio Negro'	48.3 ± 6.01 a	58.4 ± 5.80 a
H. × *chmielii* 6 × 'Gervase'	72.7 ± 12.02 a	73.8 ± 10.88 a
H. × *chmielii* 6 × 'Royal Velvet'	75.4 ± 6.28 a	77.9 ± 4.62 a

*Data after Bliss transformation.

**Means ± standard deviation in a row followed by the same letter does not differ significantly at $p = 0.05$.

CONCLUSIONS

1. The results indicate the possibility of propagation by seeds of *H.* × *chmielii*, which is confirmed by the assessment of the receptivity of the stigmas and ovules of both tested clones (No. 6 and No. 18).
2. As a result of pollination of the flowers of *H.* × *chmielii* with viable pollen of three selected cultivars of *H. hybridum*, viable seeds were obtained in most of the studied combinations, which confirms the possibility of using the *H.* × *chmielii* as a maternal form in further breeding.
3. Seeds obtained by successful cross-breeding of *H.* × *chmielii* with *H. hybridum* were characterised by a shorter (by 1 week) ripening period for the standards given in the literature for the hybrid cultivars.
4. Both tested germination methods were positive, but germination in water in jars proved to be more effective for one of the cross-breeding (*H.* × *chmielii* clone No. 18 × *H. hybridum* 'Gervase') compared to germination on Petri dishes with filter paper soaked in distilled water.

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AUTHOR CONTRIBUTIONS

P. M. contributed in this paper by conducting experiments while P. M. and D. S. by writing the manuscript. A. J. and D. S. contributed their services in research designing. All authors read and approved the final draft of the manuscript.

CONFLICT OF INTEREST

All the authors declare that there is no conflict of interest.

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