Adv. Hort. Sci., 2017 31(1): 45-51 DOI: 10.13128/ahs-20725

Development of pollination and *in vitro* germination techniques to improve the hybridization in *Hydrangea* spp.

G.A. Venturieri [1], B. Nesi [2], S. Lazzereschi [2], S. Pecchioli [2], G. Burchi [2]

[1] *UFSC/CCA-FIT, Federal University of Santa Catarina, Campus Itacorubi, Rod. Admar Gonzaga, 1346 Itacorubi, CEP 88034-000, Florianopolis (SC), Brazil.*

[2] *Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Unità di Ricerca per il Vivaismo e la Gestione del Verde Ambientale ed Ornamentale (CREA-VIV), Via dei Fiori, 8, 51017 Pescia (PT), Italy.*

Key words: breeding, fertilization barriers, fruit cut system, hortense, seed disinfection, sowing system.

Abstract: *Hydrangea* is a genus of ornamental plants which is gaining new markets mainly as a fresh or dried cut flower, but it is also important as a pot plant and for landscaping. To expand its market, new hybrids should be developed. To increase the hybridization efficiency, some techniques were developed and tested: i) evaluation of two pollination systems; ii) comparison among fruit-cut systems before *in vitro* cultivation to develop embryos and to allow the growth of new genotypes; iii) evaluation of seed disinfection systems for *in vitro* germination; iv) sowing systems using seeds and fruits from stocks cultivated in two environments. To increase inter- and intra-specific hybridization, pollination by dispersion of previously collected pollen on the top of a corymb by a brush was more effective than pollination using the corymb itself as a brush. A longitudinal cut system can be considered the best treatment to be applied on fruits before *in vitro* cultivation to allow growth of seedlings. Sterilization of seeds can be done by immersion in a solution of commercial bleach for 5 minutes on MS culture medium with PPM®. When stocks are cultivated in greenhouses, *in vitro* contamination is lower and seeds have a better rate of germination. The results of these experiments were applied in a breeding program on *Hydrangea* using sexual crosses.

1. Introduction

The genus *Hydrangea* L. includes 23 species, mainly distributed in the American and Asiatic continents (McClintock, 1957). It is a very popular ornamental plant for both garden and interior design and has recently been commercialized as a high value fresh or dried cut flower.

Interest in *Hydrangeas* is mainly due to the striking coloration of its inflorescences (corymbs or panicles), that range from pink, blue, white, to light purple or dark purple. Flowers are produced from early spring to late autumn and have two inflorescence morphologies: 'mophead' - with large flowers forming spherical flower heads; and 'lacecap' that resemble round, flat flower heads with a center core of subdued, fertile flowers surrounded by outer rings of showy, sterile flowers.

To further increase their popularity, new hybrids and cultivars should be developed. In flowering plants, the main objective of selective breeding is to increase genetic variability in ornamental traits such as flower color, flower shape and plant form. To achieve this objective, intra- and inter-specific hybridizations have been widely used in breeding programs. Hybrids between *H. macrophylla* (Thunb.) Ser. and *H. paniculata* Sieb. were produced using embryo rescue, but the resulting plants were sterile and lacked vigor (Reed *et al.*, 2001; Reed, 2004). *In vitro* embryo rescue procedures have been used to facilitate the recovery of interspecific hybrids of many genera (Bridgen, 1994; Sharma *et al.*, 1996), and have recently been used to recover a putative *H. macrophylla* (Thunb.) Ser. x *H. arborescens* L. hybrid (Kudo and Niimi, 1999 a, b). Hybrid embryos often resume growth and develop into normal plants when removed from ovules and placed on an aseptic nutrient media. This procedure is known as in ovule

(*) Corresponding author: giorgini.venturieri@ufsc.br
Received for publication 5 October 2016
Accepted for publication 15 February 2017

embryo culture (Reed, 2000).

In vitro germination of seeds is a common technique used to overcome incompatibility barriers in ornamental plant hybrids (Eeckhaut *et al.*, 2006 a, b; Lazzereschi *et al.*, 2012; Nesi *et al.*, 2012). Since most *Hydrangea* seeds are so small (diameter of 0.5 mm) and hybrid seeds production is usually low (0-5 seeds/fruit), so, the development of a successful *in vitro* method for *Hydrangea* seed germination would be an important tool for breeding programs.

To increase seed germination, Greer and Rinehart (2010) have developed an *in vitro* method for cultivation and assay of *H. macrophylla* (Thunb.) Ser. and *H. paniculata* Sieb. seeds, through germination on solid media in conjunction with Plant Preservative Mixture (PPM®, Plant Cell Technology, Inc., Washington, DC, USA), and by sterilizing seed with trichloro-s-triazinetrione (Trichlor).

The objective of the present study was to develop and test several techniques for the generation and recovery of hybrids in *Hydrangea*. These included: two pollination systems (using a brush or an inflorescence on the top of the corymb); different ovary cut systems, aimed at embryo and seed rescue; different seed disinfection systems for *in vitro* germination (evaluating the time of immersion in a bleach solution and the addition of Plant Preservative Mixture - PPM® in media); comparison among three sowing systems using seeds and immature fruits in aseptic conditions, and in compost in climatized beds, from stocks cultivated in two environments (in a greenhouse and under shading net). The outcomes of these experiments were used to determine best practices for the hybridization of *Hydrangea* species using sexual propagation.

2. Materials and Methods

The experiments were carried out at the Council for Agricultural Research and Economics - Landscaping Plants and Nursery Research Unit (CREA-VIV) in Pescia (PT) (43° 49' 00" N; 10° 48' 00" E), Italy. At CREA-VIV, a germplasm collection of *Hydrangea*, composed of 66 genotypes belonging to *H. macrophylla* ssp. *macrophylla* (Hortensia and Lacecap Group), *H. paniculata, H. serrata, H. villosa, H. quercifolia, H. anomala* ssp. *petiolaris, H. arborescens, H. heteromalla, H. involucrata, H. aspera* and three genotypes of *Schizophragma hydrangeoides*, was maintained. Some trials were also arranged in Sanremo (IM) (43°49' N; 7°47' E) with some genotypes replicated from the CREA-VIV collection. Selected cultivars of this collection were used for hybridizations. During the summer of 2014, crosses among different genotypes, belonging to *H. macrophylla* ssp. *macrophylla* (38 cultivars), *H. macrophylla* ssp. *serrata* and *H. paniculata* (5 cultivars each); *H. arborescens*, *H. aspera*, *H. quercifolia* and *H. involucrata* (one cultivar for each species) were made (Table 1). Then, the capsules obtained from controlled pollinations were collected and used as starting material in the experiments, as described below.

Table 1 - Plant material used for intra- and inter-specific crosses between different genotypes of *Hydrangea* spp.

Species	Cultivars
H. macrophylla ssp. *macrophylla*	'Alberta', 'Alpen Gluhen', 'Ayesha', 'Benxi', 'Bianca Ceriana', 'Dienemann', 'Elbatal', 'Endless Summer', 'Etoille Violette', 'Europa', 'First Red', 'Grattino', 'Green Shadow', 'Hanaby', 'Harlequin', 'Intermezzo', 'Lake San Markos', 'Lanarth White', 'Lemon Wave', 'Libelle', 'Magical Coral', 'Magical Garnet', 'Magical Jade', 'Magical Noblesse', 'Masja', 'Myharayama Yae', 'Nymphe', 'Paris', 'Red Beauty', 'Renate Wate', 'San Baronto', 'Schnball', 'Seour Therese', 'Sibilla', 'Tricolor', 'White First', 'Zorro' and 'Kardinal'
H. macrophylla ssp. *serrata*	'Acuminata', 'Blue Bird', 'Miranda', 'Preziosa' and 'Yae-no-amacha'
H. paniculata	Limelight', 'Phanthom', 'Pink Diamond', 'Unique', and 'Vanilla Fraise'
H. arborescens	'Annabelle'
H. aspera	'Rowellane'
H. involucrata	'Yorakutama'
H. quercifolia	'Snow Queen'

Pollination systems

Twelve crosses, involving *H. macrophylla*, *H. arborescens* and *H. quercifolia,* randomly distributed in a germplasm collection, were subjected to two pollination systems (treatments). Before pollination, sterile flowers and all extremely immature fertile were removed from inflorescences to be used as females prior to opening of the fertile flowers. After, the petals and anthers of all remaining fertile flowers were removed and the inflorescence was covered with a breathable plastic bag. Inflorescences to be used as males were also bagged prior to flower opening. Pollination experiments were performed 1 to 4 days following emasculation.

The two pollination treatments included: a) pollination by the dispersion of previously collected pollen on the top of a corymb, aided by a brush; b) a simple dispersion of pollen using the corymb as a brush, where anthers presenting freshly-dehisced pollen were touched with the exposed stigmas of the

emasculated flowers. After pollination, inflorescences were covered again with the bags which remained on the plants until fruit collection. Effect of treatment was evaluated by the number of developing fruits on bagged female flowers. A *t* test was used to compare averages between treatments (Sokal and Rohlf, 1981).

In total, one hundred and thirty-five hand pollinations were carried out in Pescia, and 33 in Sanremo, using a different subset of *Hydrangea* species. Each species cross was repeated 3 times.

Embryo and seed rescue from immature fruits

Approximately 90 days after pollination (DAP), well-developed fruits (n = 444), obtained from the breeding program were used to investigate rates of embryo and seed rescue from immature fruits.

Fruits were sterilized in a solution of commercial bleach (5% of active chlorine), in distilled water (1:2, v/v) plus 2 µl/100 ml Tween20® (Sigma, St. Louise, MO, USA) for 10 minutes. Subsequently four ovary cut systems (treatments) were applied: a) stigma off - stigmatic branches were removed; b) longitudinal cut - stigmatic branches were removed and a longitudinal cut, from the top of the fruit up to approximately to peduncle insertion, was made; c) equatorial cut - stigmatic branches were removed and a transversal cut at the larger diameter of the fruit was made (both sections were cultivated); d) top cut - 1/3 of the distal part of the fruit was removed (Fig. 1 a-d).

Fruits were cultured in a one-half strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 1 mg/L of naphthalene acetic acid (NAA), 1 ml/L of PPM®, 30% sucrose, 6 g/L of agar and pH was adjusted to 5.8. Fruits were individually placed in test tubes. Six replication were used by cross. All vials were cultivated in a chamber maintained at 23±1°C under 16 h/day photoperiod provided by fluorescent tubes at 35 µmol/m·s.

Ovaries that were considered as contaminated or dead were counted 7 days after, and their proportion (*p*) was a square root of transformed arcsine (Ayres *et al*., 2007) and evaluated using Analysis of Variance. Averages were compared by Fisher LSD test of significance for α= 0.05 (Ayres *et al*., 2007). Values were expressed as a percentage. Physical attributes of germinated seedlings were described for each treatment.

Disinfection of seeds for in vitro *germination*

Fruits considered as mature (130±10 DAP) were collected and left to dry on a laboratory bench, and then grinded carefully using a mortar and pestle to liberate seeds from capsules. The obtained mass was passed through 0.71 mm mesh of a soil gradation sieve (Grade 25, Giuliani Tecnologie, Scientific Instrument, Torino, IT) to remove large debris, and used in two disinfection experiments as described below.

Sterilization as a function of time immersion in bleach solution. About 0.025 g of the mass of seeds and debris was placed in a piece of TNT envelope and submerged for the disinfection process (Fig. 2 a). The envelopes were dipped in 70% ethanol for 30 sec, followed by sterilization in a commercial bleach solution, as already as described above, for 5, 10, 20, and 30 minutes and rinsed in sterilized distilled water twice (Fig. 2 b). Then the envelopes were opened (Fig. 2 c) and their contents were laid on a one-half MS culture medium supplemented with 1 mg/L of NAA, 1 ml/L of PPM®, and 30% sucrose, and adjusting pH to 5.8. TNT was used to cover petri dishes (Fig. 2 d), and then cultures were placed in the chamber as described above. Germinated seeds and contamination of petri dishes were evaluated after 15 days. Frequency of contaminated and uncontaminated petri dishes that contained germinated seeds was used to evaluate the effect of contamination on ger-

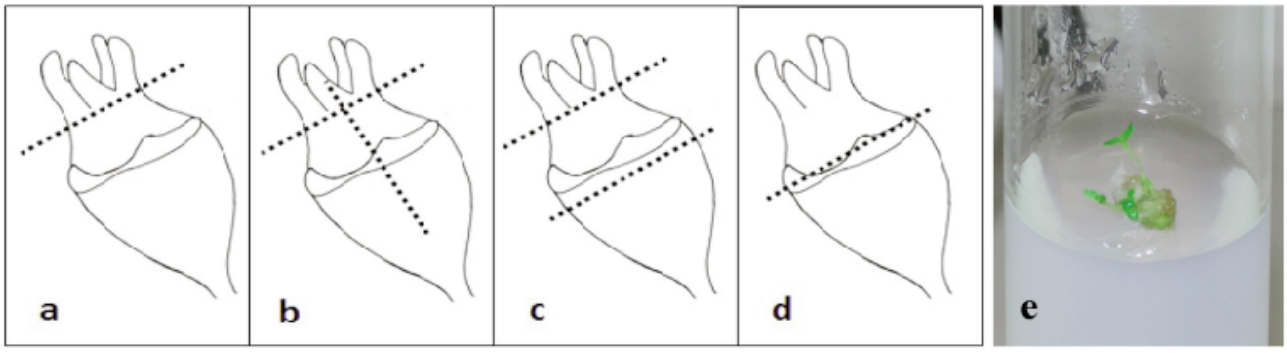

Fig. 1 - Graphic representation of the different cuts applied to the immature fruits of Hydrangea: a) stigma off; b) longitudinal cut; c) equatorial cut; d) top cut (Fig. 1 a-d); e) development of new plantlet from immature fruits *in vitro* cultured.

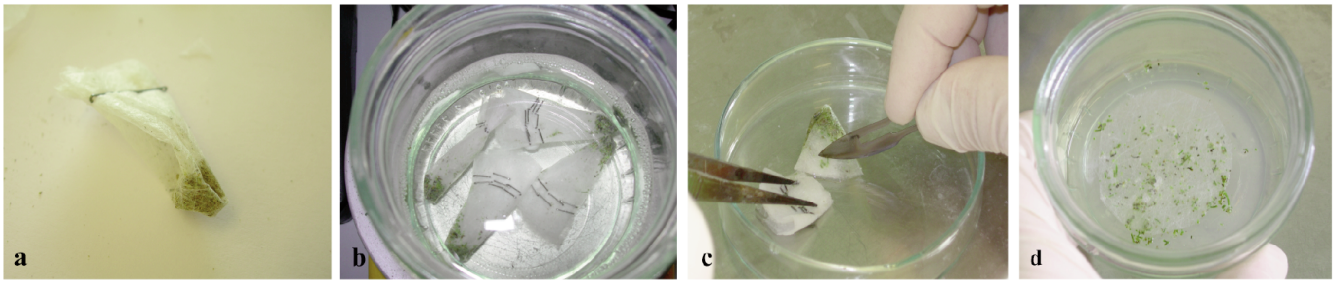

Fig. 2 - Disinfection process of seeds obtained from mature capsules harvested: (a) TNT envelops with seeds plus debris inside; (b) Envelops dipped in the disinfection solution; (c) Staples were taken off and envelop opened; (d) Seeds plus debris were layered on medium and TNT was left to cover seeds.

mination by Fisher's test. Spearman rank order correlation was calculated between number of germinated seeds and disinfection time (Ayres *et al*., 2007).

Sterilization as a function of duration in bleach solution and PPM® concentration in the media. In this experiment, culture medium and *in vitro* sowing system were the same as described above, but using a factorial design of two variables: PPM® in the medium at 3 different concentrations (2, 4 and 6 ml/L) and four disinfection times (5, 10, 20 and 30 minutes). About 0.025 g of the mass of seeds was utilized to the disinfection process. Each treatment was repeated 3 times. The number of germinated seeds per petri dish and the proportion of contaminated petri dishes were used as parameters. A factorial analysis of variance was performed and averages were compared by Fisher LSD test of significance for α=0.05.

Sowing systems using seeds and fruits from stocks cultivated in two environments

Seeds were sown in Pescia, using material from two environments (Pescia and Sanremo), following three different systems. From 130 different crosses made in Pescia, seeds from 33 crosses were sown *in vitro,* well developed but still immature fruits, from 22 crosses were cultivated *in vitro* and seeds from 130 crosses were sown in organic compost (peat and perlite, 1:2, v/v) on artificially climatized beds, inside a greenhouse. From 34 different crosses made in Sanremo, seeds from 19 crosses were sown *in vitro*, well developed but still immature fruits from 24 crosses were cultivated *in vitro* and seeds from 34 crosses were sown in the same compost and conditions as described above. About 0.025 g of the mass of seeds was utilized to *in vitro* sowing for each combination of cross. Three ovaries of each cross were *in vitro* cultivated, and of the total mass of seeds were sown in organic compost. Each sowing system was repeated 3 times.

All *in vitro* germinations were carried out in fall-winter of 2013 and compost germination in summer of 2014.

Due to the difficulty in counting the number of seeds sown inside a fruit or inside a mass of sieved seeds and debris, the comparison was based in number of germination events that happened per treatment. A nonparametric χ^2 statistical analysis was applied, and where significant intergroup differences were found, multiple comparisons were conducted using the partitioning χ^2 test to differentiate between treatments, both for α=0.05 (Ayres *et al.*, 2007).

3. Results

Pollination systems

According to the *t*-test, pollination using a brush with pollen collected from the corymb (average of 7.6 fruits per inflorescence cross), showed a mean significantly higher than using a corymb as a brush (average of 4.8 fruits per inflorescence cross) (p= 0.001). Although, the time needed for pollination using a brush is considerably greater than that using only a corymb.

Embryo and seed rescue from immature fruits

No statistical significance (p=0.07) was observed in the rate of contamination between different cut treatments. A lower percentage of contamination was observed in the treatment "stigmas off" (Fig. 1 a), probably due to less damage in the fruit tissues compared to the other cutting systems (Table 2). The number of dead fruits was not affected by the different fruit cut systems applied (p=0.50). Cut system were further differentiated based on variation in germination behavior. In the treatments "stigmas off" and "top cut", fruits swelled but seedlings did not emerge from fruit, suggesting poor germination. Furthermore, some seedlings were confined inside the fruit and did not develop and grow outside of the fruit. In the "equatorial cut" treatment, two portions of the fruit were grown: seeds were able to germi-

nate and develop new seedlings from the lower portion, but not from the upper portion. This suggests a potential loss of seedlings. Conversely, the treatment "longitudinal cut" (Fig. 1 b) allowed the seeds to germinate and seedlings promptly grew and developed upright (Fig. 1 e).

Table 2 - Proportions of contaminated and dead fruits, by ovary cut system, and behaviour of eventual germinated seeds from survived fruits

Cutting systems	Sample size (N)	Contamination (%)	Death (%)	Behaviors of germinated seedlings
Stigma off	150	31 a [z]	31.3 a	Fruits swelled but seedlings could not emerge from fruit and develop
Longitudinal cut	150	34 a	38.0 a	Seeds germinated promptly, grew up and develop upright
Equatorial cut	72	38 a	29.2 a	Germinated seed developed but none from the top slices, suggesting potential seedlings loses
Top cut	72	49 a	31.9 a	Some seedlings were confined inside the fruit and did not emerged

(z) Values followed by the same letter in each column do not differ statistically for α=0.05.

Disinfection of seeds for in vitro *germination*

Sterilization as a function of time immersion in bleach solution. No statistical differences were observed in the number of contaminated petri dishes between genotypes (p=0.37) or duration of disinfection period (p=0.22). Seed germination was lower in contaminated petri dishes only, where sterilization time was positively correlated with the number of germinating seeds (r_s of Spearman=0.51; p=0.01) (Table 3).

Sterilization as a function of duration in bleach solution and PPM® concentration in the media. Seed immersion duration in bleach solution did not significantly affect the rate of contamination (p=1.00); however, a significant difference was observed for PPM® concentration (p≈0.00). No interaction between these two variables was observed (p=1.00). Therefore, seeds surface sterilization with bleach solution was not enough to prevent contamination, but sterilization was achieved only with the addition of PPM®. Contamination occurred only when 2 ml/L of PPM® were added to the culture medium; when a higher concentration of PPM® was used, no contamination was observed at any time of immersion in the solution of bleach (Table 4).

So, to sterilize seeds for *in vitro* germination, the use of a culture media with 4 ml/L PPM® and a sterilization of 5 minutes is recommended.

Table 3 - Effect of application of bleach solution in function of duration of disinfection by different *Hydrangea* genotypes

Genotype	Duration of disinfection (minutes)				Contaminated Petri dishes/ genotype
	5	10	20	30	
H. macrophylla spp. *macrophylla* Proc. Izu Ohoshima	○[z]	○	○	○	0
H. macrophylla spp. *macrophylla* Proc.Takeoka Chiba	○	○	○	○	0
H. involucrata Proc. Yamamae Yoko Tama	○	○	○	○	0
H. macrophylla 'Libelle' x *H. paniculata* 'Limelight'	●	●	●(♣)	●(♣♣♣♣♣)	4
H. involucrata 'Myharayama kokonoe Tama' x *H. macrophyll*a 'Alberta'	●	●	●	●(♣♣♣)	4
H. macrophylla 'Libelle' x *H. macrophylla* 'Europa'	●(♣)	●(♣)	●	●	4
Total of contaminated Petri dishes/duration of disinfection	3	3	3	3	

(z) Symbols: ○ uncontaminated petri dishes; ● contaminated petri dishes; ♣ number of germinated seed.

Table 4 - Evaluation of PPM® concentration on the number of *Hydrangea* spp. germinated seeds/per petri dishes, and proportion of contaminated petri dishes

PPM® concentration (ml/L)	Germinated seeds/petri dishes (N)	Contaminated petri dishes (%)
2	5.5 a [z]	66.7 a
4	3.8 a	0 b
6	4.0 a	0 b

(z) Averages and percentage (by column) followed by the same letter do not differ at α>0.001.

Sowing systems using seeds and fruits from stocks cultivated in two environments

The average percentage of germination in Pescia was 30.5%; and in Sanremo, 55.8%. Inside the environment "Pescia", no statistical differences were observed between sowing systems (p=0.8, d.f.=2), but in "Sanremo" there were differences (p≈0.00 d.f.=2). Partitioning χ^2 tests revealed that treatments in the Sanremo environment were all statistically different from each other (p<0.007, d.f.= 1), with immature fruits cultivated *in vitro* demonstrating the highest percentage of germination among treatments (Fig. 3).

4. Discussion and Conclusion

In the present paper, we use several experiments to define work strategies and priorities to hybridize

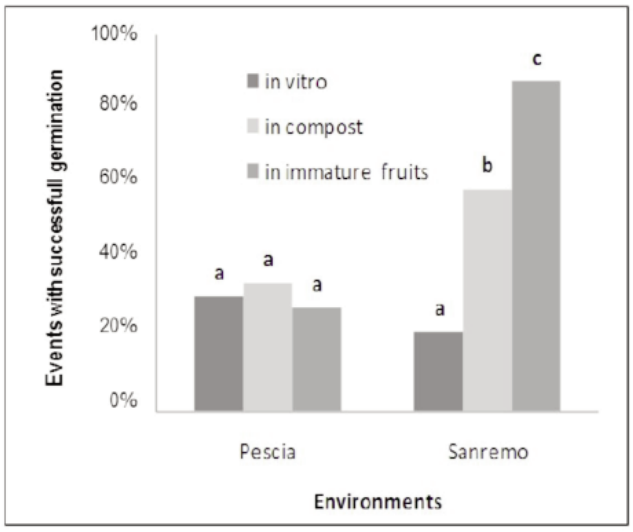

Fig. 3 - Events with successful germination of potential *Hydrangea* spp. hybrids by different sowing systems, in two environments. Columns, by environment, followed by the same letter do not differ statistically for α=0.05.

Hydrangea species using sexual propagation. The aim of the project was to hybridize all species involved in a breeding program, therefore pollination and *in vitro* germination techniques had to be developed to maximize efficiency.

To efficiently produce fruits of inter- and intraspecific hybrids, pollination by the dispersion of previously collected pollen on the top of a corymb aided by a brush resulted in better pollination than using the corymb itself as a brush. Nevertheless, pollination using only the corymb was used for the purpose of the present project because it was faster and more convenient. However, the hybrid origin should be certified using molecular markers when seedlings are established or at flowering, based on morphological characters.

Based on the germination behaviour, the "longitudinal cut" system applied to fruits prior to their transfer to the culture medium is recommended to promote vigorous growth of seedlings. It is considered as the best system because seeds readily germinated and developing seedlings promptly grew upright. Similar responses were also observed in the production of interspecific hybrids of *Lilium longiflorum* Thunb. and *L.* × *elegans* (Roh *et al*., 1996).

Seed surface sterilization for *in vitro* germination with bleach solution was not enough to prevent contamination, but sterilization was achieved with the addition of PPM®. In some plant species, germination is favoured by the presence of microorganisms, usually attributed to *Rhizobacteria* (Saharan and Nehra, 2011), but seldom adapted to *in vitro* germination of ornamental plants except in orchids (Tsavkelova *et al*., 2007); this probably could be an explanation of what happened in the case of *Hydrangea* seeds. For the objectives of the present study, however, it would not be an advantage because almost all culture media were completely covered by fungal mycelia causing seedlings to collapse within the first week following germination.

Germination in fruits showed a higher number of successful germinations than the other two systems. The highest proportion of germination success, observed for "*in vitro* inside fruit with a longitudinal cut for embryo and seed rescue" system, could be due to supplementary nutrients, given by the medium, that ensured development of hybrid seeds without endosperm (Eeckhaut *et al*., 2006 a and b). Nevertheless, specific studies of endosperm lacking in seeds from *Hydrangea* hybrid crosses are unknown.

The number of germinated seeds in Pescia was lower than in Sanremo: in Pescia the stock plants were cultivated under shading net while in Sanremo in greenhouse conditions. In fact, the contamination of explants cultivated *in vitro* can be associated to management of stock plants.

Pollination system and *in vitro* germination techniques have allowed us to obtain a number of new individuals with potential ornamental traits. Currently this material is under selection, providing the basis for the development of a *Hydrangea* breeding program in course at CREA-VIV at Pescia - Italy.

Acknowledgements

The authors thank to Drs. Francesco Ferrini and Laura Mugnai, throughout the international cooperation agreement between the Federal University of Santa Catarina - Brasil and the University of Florence - Italy; to the Council for Agricultural Research and Agricultural Economics Analysis/ Landscaping and Nursery Plants Research Unit, at Pescia - Italy, for the laboratories and support. We also thanks for the Brazilian Government, by the Program Science Without Border from CAPES/CNPq, for the grant given for the first author. And we also acknowledge Dr. Alistair Campbell for linguistic advice.

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