**Shortening of selection time of Rosa hybrida by in vitro culture of isolated embryos and immature seeds**

# SHORTENING OF SELECTION TIME OF *ROSA HYBRIDA* BY *IN VITRO* CULTURE OF ISOLATED EMBRYOS AND IMMATURE SEEDS.

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**Abstract**

*In vitro* protocols for embryo and immature seed germination may offer valuable tools to increase hybridisation efficiency, to observe genotypes of ornamental interest, and to shorten the production time of *Rosa hybrida* commercial cultivar seedlings. In the present study, rose hips were harvested weekly from 7 to 35 days after pollination (DAP). Thereafter, the immature seeds were extracted and surface disinfected. One half of them were directly plated as immature seeds onto culture medium to induce germination under several different phytohormone concentrations and culture conditions; the other half were opened and the embryos were excised and also plated onto similar culture media and conditions to germinate. Results showed that *in vitro* germination was possible for both embryos and immature seeds commencing at 21 DAP. The optimum protocol (final germination percentage greater than 40%) used embryos at 28 DAP on a medium supplemented with 2.5 mg $L^{-1}$ benzylaminopurine (BAP) and 0.5 mg $L^{-1}$ gibberellic acid ($GA_3$), which had been incubated for one week at 4 °C in dark conditions and subsequently transferred under a 16 hour photoperiod at either 23 °C or 15 °C. The protocols developed in this investigation might help breeders to improve and speed seed germination and to screen a wide morphological variability which would result in shortened selection time.

**Key words**: Breeding programs; benzylaminopurine; germination; gibberellic acid; hybrid tea roses

**Running title:** Embryo and immature seed culture to speed rose breeding

## Introduction

Hybrid tea roses (*Rosa hybrida*) are among the most economically important cut-flower plants worldwide (Schubac 2010). Rose cultivation began about 500 years ago by ancient civilizations in China, West Asia, and North Africa (Gudin 2000). Throughout its history, important characteristics such as flower colour and shape, inflorescence structure, and plant habit have been introduced from the progenitors of modern cultivars into the rose cultivar gene pool (De Cock et al. 2007). Breeders are always looking for new and novel varieties to be competitive in the flower market. Conventional breeding programs, based on sexual crossings, can be divided into three major phases: new progeny creation, seedling selection, and propagation and market introduction (Pipino 2011). In the past, selection of a new cultivar could take as many as ten years; today this has been reduced to five years, although breeding selections remain in the range of seven to eight years (Pipino 2011).

A major bottleneck in rose breeding is the low and non-uniform seed germination that limits progeny sizes and lengthens generation intervals. The main causes are the mechanical restriction of embryo expansion due to a thickened pericarp (physical dormancy), hormonal dynamics (physiological dormancy), or the premature abortion of developing embryos (Semeniuk et al. 1963, Gudin 1994, Mohapatra and Rout 2005). Thus, achieving uniform and high rates of germination in short time has long been a challenge for rose breeders (Zlesak 2006).

Botanically, rose seeds are achenes, i.e., dry, monocarpellate, indehiscent fruits, consisting of a woody and hard pericarp, seed coat (*testa*), and embryo. Despite that, "seed" will be used throughout the text to denote this physiological structure. In general, seeds are considered mature after four months or 120 days after pollination (DAP). This is when the hypanthium tissue just begins to change or completely changes colour (Gudin et al. 1990, Zlesak 2006). This stage is assumed to be the optimal moment for collecting hips, and correlates to a period between excessive accumulation of germination inhibitors and sufficient photosynthate translocation to the embryo (Zlesak 2006). Before sowing, seeds are commonly cold stratified at 4 °C for about two months at moderate moisture levels (Zlesak 2006).

Many attempts have been made in an effort to enhance germination. In particular, hormonal treatments such as pre-sowing in gibberellic acid ($GA_3$) have been used to improve germination rates (Tilberg 1983, Zhou et al. 2009, Pipino et al. 2011); however, they have often proved ineffective and time-consuming (de Vries and Dubois 1987; Ogilvie et al. 1991). *In vitro* techniques have also been undertaken to improve rose breeding programs (Pati et al. 2006). Numerous studies have been involved *in vitro* culture of axillary buds, shoot tips, and calli from several

vegetative and floral parts (Bressan et al. 1982, Kunitake et al. 1993, Carelli and Echeverrigaray 2002, Nikbakht et al. 2005, Ram et al. 2011). Two other options to shorten the time needed to produce seedlings may exist in embryo and immature seed culture techniques. Successful embryo culture is largely determined by three factors: embryo developmental stage, culture medium and growth conditions (Gudin 1994, Mohapatra and Rout 2005). Successful seed culture mainly depends on the inhibition of seed coat imposed dormancy factors that occurs in mature brown-coloured seeds (Kelly et al. 1992).

In roses, several studies have been performed to overcome dormancy-related problems and to improve germination by using embryo rescue techniques. Mature embryo culture has been described long ago by Lammerts (1946), Asen (1948), and Asen and Larsen (1951), but attempts were poorly defined. Immature embryos were initially used by Burger et al. (1990) and Arene et al. (1993) as explant sources for *in vitro* organogenesis and plant regeneration. Later, Gudin (1994), Marchant et al. (1994), and Mohapatra and Rout (2005) undertook immature embryo rescue in *R. hybrida*, English roses, and floribunda roses, respectively. Their studies were mainly focused on medium composition (sugar source and content, mineral salt concentration and growth hormones). Very few studies have been undertaken *in vitro* using immature seeds. Kunitake et al. (1993) regenerated plants from immature seed-derived calli of *R. rugosa* Thunb. via somatic embryogenesis. Meynet et al. (1994) reported that immature seeds at 35 DAP in *R. hybrida* 'Sonia' successfully germinated *in vitro* without being stratified.

The aim of this research was to compare *in vitro* culture of embryos and immature seeds of *R. hybrida* in the early seed development phases and then outline an efficient protocol to shorten the period from pollination to hip harvest.

## Materials and methods

### Plant material and disinfection

Manual pollinations were performed in the greenhouses of NIRP International (Novità Internazionali Rosai Protetti - Bevera, Ventimiglia (IM), Italy) during June 2010. Fifteen hybrid tea rose hips obtained from the same cross were harvested at: 7, 14, 21, 28, and 35 days after pollination (DAP). They were then stored in plastic bags at 4 °C for one week in a temperature controlled room. After this period, the hips were prepared for *in vitro* culture. A total of 3000 seeds were extracted and washed with 1% (v/v) Tween 20. Then, were rinsed in running tap water for 30 minutes, surface-disinfected using 1.8% sodium hypochlorite for 30 minutes with anchor agitation, and finally

rinsed three times in autoclaved cold distilled water. One half of the seeds were immediately cultured. The other half of seeds were carefully excised in aseptic conditions using a forceps and scalpel under a stereo-microscope INTEL SINT WILD M 3Z (Leica, Heerbrugg, Switzerland) to extract the embryos.

*In vitro* germination

Seeds and excised embryos were singularly cultured into 'De Wit' tubes (Duchefa Biochemie, Haarlem, The Netherland) containing 12.0 ml of medium. Two variations of the medium were used: (1) Murashige and Skoog (1962) macro- and micro-elements including vitamins (Duchefa Biochemie, Prod. Number M02220001, 4.4 g $L^{-1}$) , and sucrose (30 g $L^{-1}$), and solidified with Gelrite (4 g $L^{-1}$) medium (defined in the text as MS0) and (2) MS0 supplemented with benzylaminopurine (BAP, 2.5 mg $L^{-1}$) and gibberellic acid ($GA_3$, 0.5 mg $L^{-1}$) (defined in the text as MS). The pH was adjusted to 5.7 prior to addition of Gelrite and sterilized at 121 °C for 15 minutes. Germination occurred in growth chambers (60% R.H. and 55 µmol $m^{-2}s^{-1}$ under cool, white fluorescent lamps) at six different conditions: (A) 24-h photoperiod for two weeks followed by 16-h photoperiod for all the experiment, both at 23 °C; (B) two weeks dark period followed by 16-h photoperiod for all the experiment, both at 23 °C; (C) 24-h photoperiod for two weeks at 4 °C followed by 16-h photoperiod for all the experiment at 23°C; (D) two weeks dark period at 4 °C followed by a 16-h photoperiod for all the experiment at 23 °C; (E) one week dark period at 4 °C followed by a 16-h photoperiod for all the experiment at 23 °C; and (F) one week dark period at 4 °C followed by a 16-h photoperiod for all the experiment at 15 °C. Twenty five seeds and twenty five embryos per DAP, medium variant and growth condition were used.

On the basis of the International Rules for Seed Testing Association (ISTA 2014), excised embryo and immature seed germination was daily recorded for a total of 210 days. The final germination percentage (FGP), half-time of germination ($T_{50}$), and germination period (GPD) were calculated using Czabator's index (1962) where FGP = total number of seeds germinated ÷ total number of seeds in all replicates x 100; $T_{50}$ = days from seeding to the 50% of the total germinated seeds, and GPD = days from seeding to when maximum number of seeds germinated. Moreover, the day of the first emergence (Em) and the germination energy (Ge) were counted. Germination energy is defined as the germination percentages when the mean daily germination (cumulative germination percent divided by the time elapsed since sowing date) reached its peak. The explants (immature seeds and embryos) were considered to have germinated when cotyledons emerged. A control was established. It contained 150 mature seeds

from the same cross which were extracted from hips harvested after 120 DAP. These seeds were cold stratified at 4 °C for two months and placed in perlite seed beds for germination in the greenhouses of the NIRP International.

Statistical analysis

Arcsine transformation was performed on all percent incidence data (germinated embryos and immature seeds, and germination energy) before statistical analysis in order to improve homogeneity of variance. Effect of DAP, explant type, medium, and growth conditions on FGP, Em, $T_{50}$, GPD, and Ge were evaluated by the analysis of variance (one-way ANOVA) using Ryan-Einot-Gabriel-Welsch's multiple stepdown F (REGW-F) test ($P \leq 0.05$). All analyses were performed with SPSS 17.0 Inc. software (Chicago, Illinois, USA).

**Results**

Germination was not observed in either the embryos or immature seeds at 7 and 14 DAP cultured under all growth conditions, nor at 21, 28, and 35 DAP cultured under growth conditions A, B, C, and D (FGP < 2%), as opposed to embryos and immature seeds cultured under growth conditions E and F (Fig. 1). In these latter conditions, germination indexes (FGP, Em, $T_{50}$, GPD and Ge) were affected by developmental stage, explant type, medium variant, and growth conditions as showed in Table 1. The protocols yielding significant better outcomes (with FGP ≥ 40%) resulted from the following parameters and sequencing: *in vitro* culture of embryos collected at 28 DAP on MS, incubation in darkness at 4 °C for one week; germination under a 16 hour photoperiod at either 23 °C (E) or 15 °C (F). These protocols yielded 40% and 48% FGP, respectively, resulting significantly much higher than control, i.e., seeds germinated conventionally in the nursery greenhouse (7.6% FGP). In these *in vitro* conditions the highest Ge percentages (15.9% and 17.4%, respectively) occurred. The lowest Em, $T_{50}$ and GPD were observed in embryos collected at 35 DAP and cultured on MS0 under both growth conditions and on MS under growth condition E (days 21, 25 and 25; days 29, 32 and 31; and days 36, 40 and 40, respectively). On the opposite, the highest Em, $T_{50}$ and GPD were observed in seeds at 28 DAP, cultured on MS0 under growth condition F or cultured on MS under growth condition E (days 140 and 150; days 167 and 163; and days 182 and 182, respectively). Seeds at the same age, cultured on MS0, and under growth condition F also showed the lowest Ge (0.8 %).

**Discussion**

Previous studies suggested that the optimal time point for performing embryo rescue in hybrid roses could be comprised between 15 and 30 DAP, i.e., when embryos are completely developed, the ABA concentration is significantly reduced but the seed pericarp is not yet completely hardened (Pipino et al. 2013, Bosco et al. 2014).

In agreement with this information, in our study neither the embryos nor the immature seeds harvested at 7 and 14 DAP germinated. Germinations started to occur with explants harvested at 21 DAP, one to three weeks earlier than in the protocols proposed by Gudin (1994) and Mohapatra and Rout (2005).

Continuous light (24-photoperiod) and constant temperatures (23 °C) negatively affected germination, which occurred only in cultures incubated for one week in the dark at 4 °C and then transferred to light (16-photoperiod) and 23 °C or 15 °C (growth conditions E and F). In the dark and cold conditions, a lower production of polyphenols from the surrounding tissues of the excised embryos could help in better response (Rout et al. 1999). In contrast, a prolonged period of dark (2 weeks) at cold negatively influenced germination. This was probably due to the fact that seeds collected between 21 and 35 DAP do not require excessive cold treatment because endodormancy is already fully reduced (Pipino et al. 2013). Furthermore, at this stage, the not-yet-fully lignified pericarp is unable to protect the embryo from external factor damage, such as two weeks of cold (Billings and Mooney 1968, Bousewinkel and Bouman 1995, Scheiber and Robacker 2003). Medium supplementation with BAP (2.5 mg $L^{-1}$) and $GA_3$ (0.5 mg $L^{-1}$) even further increased embryo germination rates, in agreement with Mohapatra and Rout (2005).

Overall, the protocol appears to be an attractive tool to obtain new genotypes by reducing production time. Contamination, which can seriously limit the use of embryo rescue (Arunachalam and Kaicker 1994), was less than 22%. However, even if mechanical dormancy is reduced in immature seeds (Tillberg 1983, Guzicka et al. 2012), equipment and personnel investment is likely necessary to excise large quantities of seeds. *In vitro* immature seed culture may be a more practical method for rose propagation. Using the best protocol, immature seeds harvested at 21 DAP and cultured on MS under growth condition E, we obtained a FGP equal to 31.5% with a Ge equal to 9.7 % and GPD of 150 days. While the results are less efficient than embryo rescue, they are still far superior than conventional company practices (FGP = 7.6%). Moreover, the method is easy, fast, and requires no microscope or instruments to extract very soft embryos.

In conclusion, the *in vitro* systems developed here might allow breeders to obtain higher seed germination rates and to observe morphological variability faster, which results in shortened selection time. Better characterisation of embryo development and maturation might lead to a better understanding of the mechanisms

behind these processes.

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## Tables

**Table 1** Effect of explant age (DAP = days after pollination), explant type (embryo and immature seed), medium (MS0 and MS), and growth conditions (E = one week dark period at 4 °C followed by a 16-h photoperiod for all the experiment at 23 °C; F = one week dark period at 4 °C followed by a 16-h photoperiod for all the experiment at 15 °C) on final germination percentage (FGP), first emergency (Em), half-time of germination ($T_{50}$), germination period (GPD), and germination energy (Ge) in *Rosa hybrida*.

| DAP | Explant | Medium | GC | FGP *(%)* | Em *(d)* | $T_{50}$ *(d)* | GPD *(d)* | Ge *(%)* |
|---|---|---|---|---|---|---|---|---|
| 21 | embryo | MS0 | E | 10.0 (± 2.5) jklm[§] | 37 (± 4) gh | 42 (± 10) fgh | 50 (± 5) fg | 3.2 (± 0.5) de |
| | | | F | 10.0 (± 2.4) jklm | 65 (± 5) e | 70 (± 8) ef | 75 (± 11) de | 3.4 (± 0.5) de |
| | | MS | E | 33.5 (± 1.5) bcd | 41 (± 5) fg | 50 (± 10) fg | 60 (± 8) ef | 11.2 (± 0.2) b |
| | | | F | 12.0 (± 1.8) hijklm | 41(± 4) fg | 50 (± 10) fg | 60 (± 5) ef | 3.2 (± 0.8) de |
| | seed | MS0 | E | 18.5 (± 2.0) fghij | 100 (± 6) c | 118 (± 4) d | 121 (± 6) c | 5.6 (± 1.5) cd |
| | | | F | 11.5 (± 2.1) hijklm | 65 (± 3) e | 70 (± 6) ef | 80 (± 7) de | 3.5 (± 0.7) de |
| | | MS | E | 31.5 (± 0.5) bcde | 120 (± 5) b | 134 (± 4) c | 150 (± 5) b | 9.7 (± 1.2) bc |
| | | | F | 16.5 (± 1.1) ghijkl | 80 (± 4) d | 85 (± 7) e | 98 (± 5) de | 5.2 (± 0.8) de |
| 28 | embryo | MS0 | E | 24.0 (± 1.6) cdefgh | 35 (± 6) gh | 46 (± 11) fgh | 52 (± 6) fg | 7.0 (± 1.2) bcd |
| | | | F | 36.0 (± 0.9) abc | 35 (± 6) gh | 49 (± 9) fg | 59 (± 4) ef | 14.8 (± 1.5) ab |
| | | MS | E | 40.0 (± 4.0) ab | 35 (± 5) gh | 50 (± 6) fg | 59 (± 5) ef | 15.9 (± 1.0) a |
| | | | F | 48.0 (± 4.5) a | 37 (± 5) gh | 57 (± 8) fg | 65 (± 7) ef | 17.4 (± 1.2) a |
| | seed | MS0 | E | 10.0 (± 2.5) jklm | 80 (± 4) d | 99 (± 5) e | 110 (± 8) cd | 3.6 (± 0.7) de |
| | | | F | 3.5 (± 2.8) lm | 140 (± 5) a | 167 (± 3) a | 182 (± 5) a | 0.8 (± 0.2) f |
| | | MS | E | 17.0 (± 3.5) fghijk | 150 (± 5) a | 163 (± 3) a | 182 (± 5) a | 4.5 (± 0.5) de |
| | | | F | 22.1 (± 2.8) defgh | 132 (± 4)ab | 148 (± 5) b | 159 (± 5) b | 6.1 (± 0.5) cd |
| 35 | embryo | MS0 | E | 26.0 (± 4.7) cdefg | 21 (± 12) h | 29 (± 5) h | 36 (± 4) g | 8.1 (± 1.2) bc |
| | | | F | 34.0 (± 2.5) bc | 25 (± 9) h | 32 (± 4) h | 40 (± 6)g | 14.2 (± 1.5) ab |
| | | MS | E | 22.0 (± 3.9) efghi | 25 (± 9) h | 31 (± 4) h | 40 (± 6) g | 6.5 (± 0.8) cd |
| | | | F | 30.0 (± 2.5) bcdef | 46 (± 4) fg | 59 (± 5) fg | 65 (± 6) ef | 9.5 (± 0.7) bc |
| | seed | MS0 | E | 0.0 (± 0) m | - | - | - | - |
| | | | F | 0.0 (± 0) m | - | - | - | - |
| | | MS | E | 7.0 (± 2.6) jklm | 142 (± 8) a | 160 (± 6) a | 172 (± 8) ab | 1.7 (± 0.5) e |
| | | | F | 0.0 (± 0) m | - | - | - | - |
| control | | | | 7.6 (± 3.5) jklm | 105 (± 5) c | 120 (± 6) d | 125 (± 5) c | 2.5 (± 0.8) de |
| | | | *P* | ** | * | * | * | ** |

The statistical relevance of 'Between-Subjects Effects' tests (*=$P$<0.05, **=$P$<0.001, ns=not significant). [§]Mean values with standard error in parenthesis followed by the same letter are not statistically different at $P$≤0.05 according to the REGW-F test.

**Figure captions**

**Fig. 1.** *In vitro* germination of *Rosa hybrida* embryo collected after 28 days after pollination (DAP), cultured on modified Murashige and Skoog medium supplemented with BAP (2.5 mg L-1) and $GA_3$ (0.5 mg L-1) (MS), incubated in the dark at 4 °C for one week and later transferred at 15 °C with a photoperiod of 16 h of light.

**Figure 1**