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# Floral characteristics and gametophyte development of *Anemone coronaria* L. and *Ranunculus asiaticus* L. (Ranunculaceae)

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ABSTRACT

*Anemone coronaria* and *Ranunculus asiaticus* both belong to the Ranunculaceae, a large plant family with many ornamentals of horticultural importance. In this study, the floral characteristics and the development of reproductive structures of these two cut flowers are investigated. On a macro-level, flower morphology was analysed by dissections, light microscopy and scanning electron microscopy (SEM). The female and male gametophyte development as well as embryogenesis was investigated on a micro-level by light microscopy of paraffin sections and fluorescence microscopy. These analyses confirmed that anthers of *A. coronaria* and *R. asiaticus* are tetrasporangiate and that the stamens of *R. asiaticus* can be transformed to petals. Pollen development in *A. coronaria* and *R. asiaticus* is of the simultaneous type. The mature pollen is spheroid, polyporate and bicellular. For both species, the development of the embryo sac is of the octonucleate *Polygonum*-type. The two polar nuclei inside the embryo sac fuse before fertilization while two of the three antipodal cells persist after fertilization. On the moment of fruitlet shedding, the embryos are not fully developed yet implicating an autonomous maturation prior to germination. Finally, pollination tests revealed that *R. asiaticus* 'Alfa' is self-incompatible. The results presented here provide new insights in the reproductive biology of *A. coronaria* and *R. asiaticus* supportive for future breeding strategies.



## 1. Introduction

The Ranunculaceae are a moderately large family with 59 genera and circa 2500 species (Tamura, 1995a). Most members of the Ranunculaceae are perennials and mainly herbaceous geophytes (Tamura, 1995b). Many genera of the Ranunculaceae have large and colourful flowers and are cultivated as ornamentals. Others contain pharmacological substances and are used as medicinal plants (Tamura, 1993; Hegnauer, 1995).

The genus *Anemone* consists of circa 150 species distributed in temperate zones in the northern and the southern hemisphere (Tamura, 1995c). It is divided into three sections: *Anemone*, *Anemonospermos* and *Pulsatilloides* (Hoot and Reznicek, 1994). The section *Anemone* is subdivided into two groups: the *Coronaria* group and the *Baldensis* group (Hoot and Reznicek, 1994). The cultivars of *Anemone coronaria* belonging to the *Coronaria* group are important for cut flower production (Laura et al., 2006). *A. coronaria* is a winter-flowering perennial which flowers from February till May in the Mediterranean region (Meynet, 1993a).

The genus *Ranunculus* consists of 600 species, making it the largest genus within the Ranunculaceae (Tamura, 1995c). *Ranunculus* is distributed in all continents, but mainly in non-tropical regions (Tamura, 1995c). In respect to ornamental value, *Ranunculus asiaticus* is by far the most cultivated *Ranunculus* species (Meynet, 1993b), especially in countries surrounding the Mediterranean Sea, where it is an important cut flower with blooming period ranging from February till April (Beruto and Debergh, 2004; Meynet, 1993b; Wicki-Freidl, 1988).

*Abbreviations:* DAPI, 4′,6-Diamidino-2-phenylindole; SEM, Scanning electron microscopy.

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Both cut flowers give the Ranunculaceae a significant economic importance. This is illustrated by the statistics of the Dutch auction with a retail of 50–60 million stalks of *A. coronaria* in 2010 and of 67 million stalks of *R. asiaticus* in 2009. Moreover this does not take the direct sell into account. Besides their cut flower importance, *A. coronaria* and *R. asiaticus* are also gaining ground as potential pot plants.

Despite the large size and cosmopolitan character of the Ranunculaceae, little is known about plant morphology and floral architecture of commercial cultivars. Therefore, knowledge of the morphology of reproductive structures and the development of gametophytes and embryos is crucial when commercial cultivars are to be used in breeding research. Moreover, since the production of these plants is still based on seed, a good comprehension of the reproductive organs is necessary. To this end, this paper focuses on the morphological and reproductive characteristics of *A. coronaria* and *R. asiaticus*.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

Three different commercial cultivars of *A. coronaria* L. (grown from seed) were selected, i.e. 'Mistral Wine', 'Mistral Fucsia' (both Mistral® Line, Biancheri Creations, Camporosso Mare, Italy) and 'Wicabri Blue' (Wicabri® Line, the Netherlands). For *R. asiaticus* L., the three commercial cultivars 'Alfa' (Vitro Line Success®, Biancheri Creations, Camporosso Mare, Italy), 'Krisma' (Istituto Regionale per la Floricoltura, Sanremo, Italy) and 'Bianco Strié' (Biancheri Creations, Camporosso Mare, Italy) were chosen.

Rhizomes were planted in September and grew until the end of April. The tuberous rhizomes were soaked in water for 24 h at 10 °C and subsequently planted in a peat-mixture ($pH_{H_2O}$ 6.5–7.5) with 5% perlite, 5% clay and fertilizers (NPK 14-16-18, 1 kg $m^{-3}$). The soil surface was sprayed with prochloraz (Sporgon®, 0.6 g $L^{-1}$) directly after planting, to prevent root rot by fungi. Standard nursery practices were used for watering, fertilization and pest control. Plants were grown in a greenhouse (natural photoperiod regime) with climate condition settings of 18 °C day temperature and 5 °C night temperature. During the day (8 a.m. to 4 p.m.) extra assimilation light was given (assimilation light HPI-T Plus Philips, PAR: 25–30 μmol $m^{-2}$ $s^{-1}$).

### 2.2. Macro-level flower measurements

Morphological characteristics (number of sepals, tepals, petals, stamens, carpels and fruitlets) were recorded during flowering (March–April) in ten flowers per cultivar. The pollen diameter was measured of at least 20 pollen grains per cultivar by light microscopy (Olympus IX81 and imaging software Cell$^M$). The analysed fruitlets were produced after controlled pollinations. Floral development was investigated by dissections and observed with an OLYMPUS SZX9 stereo microscope. Scanning electron microscopy (SEM) was done using a TM-1000 Tabletop microscope (HITACHI).

### 2.3. Micro-level flower measurements

In order to study flower structure and gametophyte development, floral tissues were fixed in 1:1:18 formaldehyde:acetic acid:70% ethanol (FAA). During the subsequent five days, the plant material was gradually dehydrated using a series of solutions with decreasing ethanol and increasing butanol concentrations, resulting on the fifth day in 100% butanol solution. The sixth day, butanol was supplemented with paraffin (48 h, 50–52 °C). After solidification of the paraffin, sections of 12 μm were made with a microtome (Jung AG Heidelberg). Paraffin slides were stained with fast green, safranin and haematoxylin (Johansen, 1940) and evaluated by a LEITZ GMBH Wetzlar light microscope.

Pollen of the cultivars was collected randomly between 8 and 10 a.m. from February till April. 4′,6-Diamidino-2-phenylindole (DAPI, 1.5 μL $mL^{-1}$ of stock solution (100 μg $mL^{-1}$)) staining and fluorescence microscopy were used to study male gametophyte development. To analyse the movement of vegetative and generative nuclei, pollen was incubated on species specific germination media for 72 h at 20–22 °C in dark immediately after harvest. The germination medium for *A. coronaria* consisted of 100 mg $L^{-1}$ $H_3BO_3$, 700 mg $L^{-1}$ $Ca(NO_3)_2.4H_2O$, 200 mg $L^{-1}$ $MgSO_4 \cdot 7H_2O$, 100 mg $L^{-1}$ $KNO_3$, 150 g $L^{-1}$ PEG 6000, 0.5 g $L^{-1}$ MES and a sugar content of 100 g $L^{-1}$ (pH 6.0), while for *R. asiaticus* the medium consisted of 50 mg $L^{-1}$ $H_3BO_3$, 300 mg $L^{-1}$ $CaCl_2 \cdot 2H_2O$, 100 mg $L^{-1}$ $KH_2PO_4$ and a sugar content of 150 g $L^{-1}$ (pH 5.5). The sperm nuclei were determined by staining the germinated pollen grains with DAPI (1.5 μL $mL^{-1}$ of stock solution (100 μg $mL^{-1}$)) and using fluorescence microscopy (Olympus IX81).

Pollinated carpels were harvested 56 h after pollination for aniline blue staining and fixed in 1:1:18 FAA for 24 h and subsequently macerated by NaOH (8 M) for 16 h. After being thoroughly washed in water, the carpels were stained in a 0.1% (w/v) aniline blue solution in 0.033 M $K_3PO_4$ for 3 h in the dark and analysed using fluorescence microscopy (Olympus IX81).

### 2.4. Self-incompatibility

To analyse self-compatibility in *A. coronaria* and *R. asiaticus*, seed set after self-pollination was used as a first indicator. Furthermore, an aniline blue staining was done upon selfing for all *A. coronaria* and *R. asiaticus* cultivars. The method was identical to the method mentioned in Section 2.3.

### 2.5. Data analysis

Unless another observation number (n) is mentioned, the means described in Section 3 are based on three cultivars and ten measurements per cultivar ($n = 30$). The averages are presented as mean ± standard error.

## 3. Results

### 3.1. Floral macro-morphology

*A. coronaria* has an unbranched reproductive shoot with a terminal actinomorphic flower (Fig. 1A). Three to five bracts, sessile or sometimes slightly connate at the base, form an involucre (Fig. 1B). The bracts have an entire margin at the base and are dentate to even lobed at the top. Occasionally the bracts can be petaloid (Fig. 1C). The circa nine tepals are petaloid and obovate (mean number: 9.3 ± 0.4). The stamens are numerous (mean number: 336.3 ± 12.4) with centripetal development (Fig. 1D). Young stamens and carpels are very similar (Fig. 1D inset). Using SEM it could be shown that the pollen is released from the anthers by a longitudinal slit (Fig. 1E). The free, mature pollen grains are spheroid and polyporate (Fig. 1F), 31.6 ± 0.3 μm in diameter ($n = 456$). The circa 1000 uni-ovular carpels (mean number: 996.8 ± 86.6) have a unicellular-papillate dry stigma (Fig. 1G–I) and are arranged on a conspicuously convex receptacle. The receptacle enlarges after anthesis and continues to enlarge during fruitlet formation. The fruitlets or achenes of all three cultivars of *A. coronaria*, obtained by pollination within the cultivar (mean number: 728.4 ± 70.7), are characterised by long soft curled hairs (Fig. 1J).