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Developmental Studies in the Christmas Rose (*Helleborus niger* L.)

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ABSTRACT

The Christmas rose (*Helleborus niger* L.) is a herbaceous, winter-green, perennial native to Southern Europe, which is also widely grown as an ornamental. In mild winters, the flowers may indeed appear at Christmas time, resembling wild roses with respect to size and color (white to pink). Reproductive development in the Christmas rose is characterized by an interesting aspect, uncommon in the world of flowering plants: after pollination and fertilization, the perianth develops a photosynthetic apparatus, and persists during fruit development. Unpollinated or depistillated flowers survive almost as long as their fruit-bearing neighbors, but do not pass through the complete greening process. Removal of the gynoecium also affects the shape of the flower and the length of the flower scape. The correlative signals which normally trigger and maintain these morphogenetic processes appear to include plant hormones synthesized in the developing fruit. Because of the size of its flowers, their long lifespan, and the changes induced by fruit development and maturation, the Christmas rose could become a useful research model for disentangling some of the complex interactions between developing seeds and the mother plant.

Keywords: cytokinin, gibberellin, flower morphogenesis, perianth greening, photosynthesis, plant growth regulator, plastid metamorphosis, source-sink relationship

Abbreviations: BA, N^6-benzyladenine; **4-Cl-IAA**, 4-chloroindole-3-acetic acid; **DZ**, dihydrozeatin; **DZ9G**, dihydrozeatin 9-glucoside; **DZR**, dihydrozeatin riboside; **DZRMP**, dihydrozeatin riboside-5'-monophosphate; **f. wt.**, fresh weight; **GA_x**, gibberellin A_x (x = 1, 3, 4 or 7); **HPLC**, high-performance liquid chromatography; **iP**, N^6-(Δ^2-isopentenyl)adenine; **iPR**, N^6-(Δ^2-isopentenyl)adenine riboside *alias* N^6-(Δ^2-isopentenyl)adenosine; **IAA**, indole-3-acetic acid; **iPRMP**, N^6-(Δ^2-isopentenyl)adenine riboside-5'-monophosphate *alias* N^6-(Δ^2-isopentenyl)adenosine-5'-monophosphate; **Z**, *trans*-zeatin; **Z9G**, *trans*-zeatin 9-glucoside; **ZR**, *trans*-zeatin riboside; **ZRMP**, *trans*-zeatin riboside-5'-monophosphate

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INTRODUCTION

The Christmas rose (*Helleborus niger* L.) (**Fig. 1**) has, for centuries, played a role in folklore and herbal medicine (Damboldt and Zimmermann 1965; Mathew 1989). It has, thus, long been grown in gardens and has locally become naturalized far north of its original range. The plant is also of interest to physiologists because the flowers turn green, after fertilization, and the perianth survives until seed ripening. This happens in a number of other species, as well; examples studied in detail include a number of orchids (van Doorn 1997) and the dicots *Chrysosplenium alternifolium* L., *C. oppositifolium* L. (Sitte 1974), and *Nuphar luteum* Sibth. *et* Sm. (Grönegress 1974). In the araceans *Spathiphyllum wallisii* Regel (Palandri 1967), *Zantedeschia aethiopica* Spreng. (Pais 1972; Chaves das Neves and Pais 1980a, 1980b) and *Z. elliotiana* Engl. (Grönegress 1974), it is the brightly colored spathe that turns green after anthesis. Within the above group of species, the Christmas rose with its large flowers is particularly suitable for experimental work. Also, photosynthesis in its greening perianth may substantially contribute to seed filling, because leaves are frequently absent, or not fully operational, during fruit ripening.

In most flowers, the perianth senesces and is subject to abscission, once it has played its part in the attraction of pollinators (Weiss 1991; O'Neill 1997; van Doorn 1997; van Doorn and Stead 1997). This highly regulated process involves complex structural and biochemical changes (Or-

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Invited Review

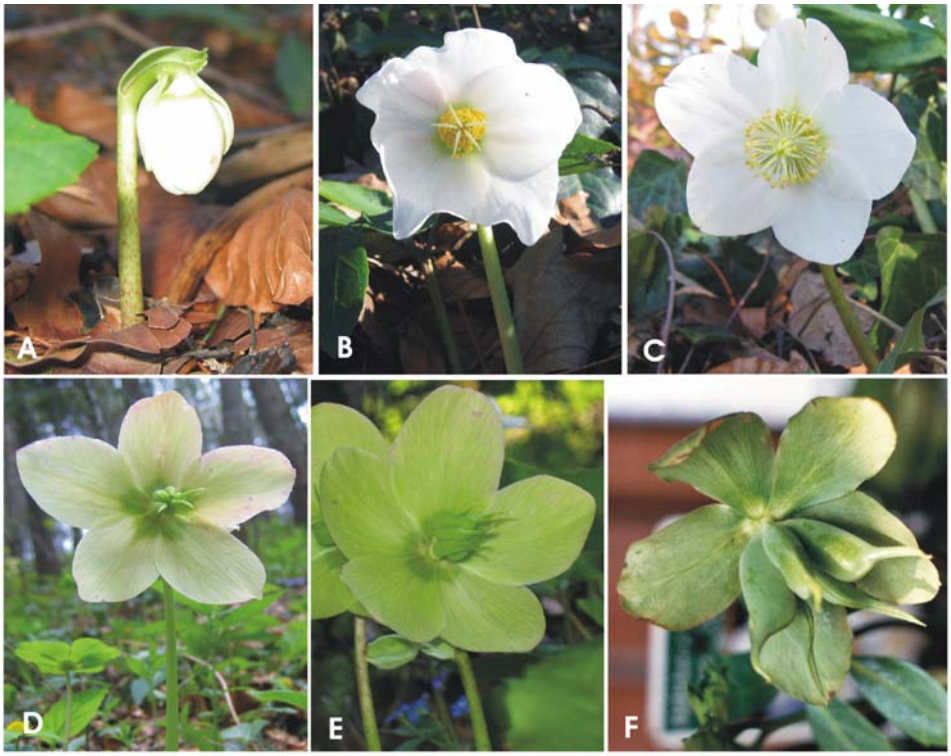

Fig. 1 Characteristic developmental stages in the life cycle of a Christmas rose flower: bud about ready to open (**A**), anthesis, female phase (**B**), anthesis, male phase (**C**), initial perianth greening (**D**), advanced perianth greening (**E**), two to three weeks before seed ripening (**F**).

záez and Granell 1997; Xu and Hanson 2000; Wagstaff *et al*. 2002; Thomas *et al*. 2003; van Doorn and Woltering 2004, 2005; Rogers 2006) and is orchestrated by plant growth regulators (Panavas *et al*. 1998; Rubinstein 2000; Wu and Cheung 2000), among which ethylene plays the first violin (Ketsa and Rugkong 1999; Orzáez *et al*. 1999; van Doorn 2002a, 2002b). The developmental mechanisms in species with a surviving perianth may reasonably be expected to be equally complex, but have so far received little attention. Here we summarize the results so far obtained studying post-anthesis development in the Christmas rose.

BOTANICAL BACKGROUND

Helleborus (*Ranunculaceae*) is a genus of spring-flowering perennials inhabiting Central and Southern Europe and the adjacent parts of Asia; one isolated species occurs in Central China (Mathew 1989). All taxonomic studies which have so far appeared were complicated by variability within, and hybridization between, species. Schiffner (1891), in his classical monograph of the genus, distinguished 17 species. In a recent revision of *Helleborus* classification, Mathew (1989) recognized 15 species, but admitted that the boundaries between some taxa are likely to remain a subject of never-ending dispute. Lately, a somewhat extended list of species has been advocated (Sun *et al*. 2001; McLewin and Mathew 2002; McLewin *et al*. 2006). The species *H. niger* L. is one of the few listed by most contemporary authors. The views on its evolutionary history experienced an interesting twist, when recent results on interspecific hybridization (Mathew 1989), nuclear DNA mass (Zonneveld 2001), shoot and seed morphology (Werner and Ebel 1994), ecdysteroid content (Dinan *et al*. 2002) and characteristic DNA sequences (Sun *et al*. 2001) indicated strong phylogenetic ties to *H. argutifolius* Viv. and *H. lividus* Aiton both of which look very different, with their elongated leafy stems topped by clusters of small greenish flowers. The relationship to other *Helleborus* species with basal leaves and large, showy flowers is, unexpectedly, not quite as close.

H. niger is indigenous to the Alps and nearby mountain ranges, preferentially on limestone or dolomite (Damboldt and Zimmermann 1965; Mathew 1989). It is a shade-loving species, but not necessarily restricted to dense woodlands. An easily accessible, detailed description may, for instance, be found in the book «The Gardener's Guide to growing Hellebores» by Rice and Strangman (1993). In brief, the plant is a herbaceous perennial with overwintering, pedately divided, basal leaves and, usually single, flowers carried on fleshy scapes which may reach up to 30 cm in height. The fully open flowers are most frequently 6–9 cm in diameter, but may easily reach 12-13 cm, not only in horticultural varieties (Rice and Strangman 1993), but also in some wild-growing Croatian populations (Šušek *et al*. 2005). The, usually five, showy elements of the Christmas rose perianth are mostly classified as sepals (Damboldt and Zimmermann 1965). They may be bluntly acuminate, rounded, or even emarginate, and their edges may, or may not, overlap, resulting in a diverse population of starry and saucer-shaped flowers in white or various shades of pink (Ravnik 1969). The whorl of sepals is followed by a ring of shortly stalked, yellowish-greenish nectaries (modified petals), a multitude of stamens, and a cluster of four to ten (most frequently five) multiovular carpels.

H. niger is a variable taxon, but only two subspecies are generally recognized (Schiffner 1891; Damboldt and Zimmermann 1965; Mathew 1989; Rice and Strangman 1993):

a) The ssp. *niger* occupies most of the range. It is most reliably identified by the shape of the leaf divisions, which are obovate to asymmetrically rhombic, widest in the distal half, and frequently edged by a few *coarse* teeth towards the tip. The plants used in the experiments described here were collected, at an altitude of 800 m, in the 'Gorski kotar' region, in an area covered by mixed forests dominated by beech (*Fagus silvatica* L.), fir (*Abies alba* L.), and spruce (*Picea abies* L.). At that locality, the soil accessible to *Helleborus* roots showed a pH of 6.1 (determined in 1 M KCl), and contained 7.20% of humus and the following macronutrients (in mg/100 g): N, 370; phosphorus as P_2O_5, 1.83; potassium as K_2O, 22.00.

b) The ssp. *macranthus* (Freyn) Schiffner has its center of distribution in Northern Italy (Mathew 1998). Schiffner (1891) also listed a number of reports for Slovenia and Croatia, which were later interpreted to mean that most, if not all, indigenous Christmas rose populations in Croatia belong to that subspecies (Martinis 1973). However, the above reports quoted in Schiffner's 1891 monograph predate his own definition of the ssp. *macranthus* (first proposed in this very monograph) and can thus not be uncritically accepted. Moreover, a number of morphological attributes (leaf color, size and shape of the sepals, length of the styles) originally

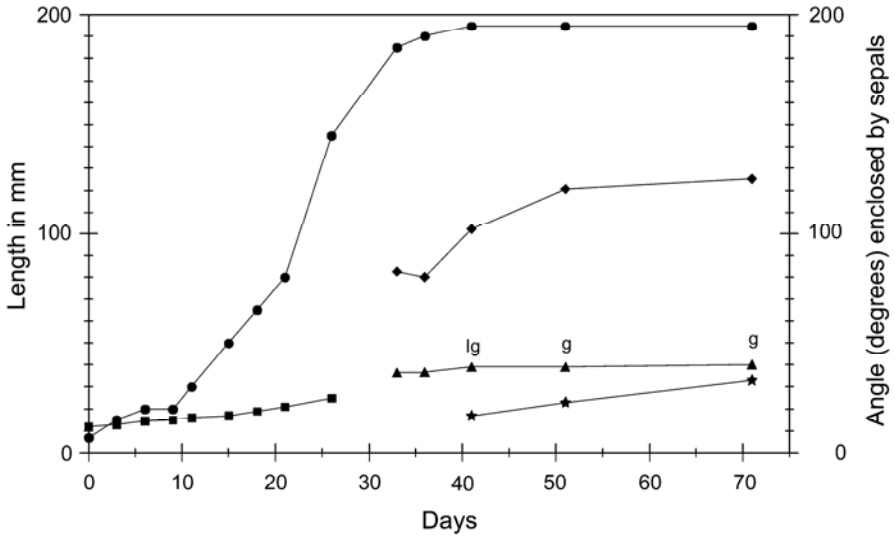

Fig. 2 Growth kinetics of a typical Christmas rose flower from the early bud stage until three weeks before seed ripening. The parameters monitored were: the length of the scape (circles), bud length (squares), sepal length (triangles), the angle enclosed by opposite sepals (diamonds), and pistil length (stars). Sepal length was determined starting at anthesis. The color of the sepals is noted above the respective data points (g, green; lg, light green; no comment, white). Measurements were taken on a potted plant of commercial origin kept on a balcony, starting February 05, 2002.

thought to characterize the ssp. *macranthus* were later found to reflect environmental impact and, likely, random genetic variability (Ravnik 1969; Šušek *et al*. 2005). What appears to be invariant is the shape of the leaf segments (broadly lanceolate, near-symmetrically tapering towards both the proximal and the distal end, edged all around with *tiny sharp* teeth). The leaves of the plants used in our experiments clearly identified them as representatives of the ssp. *niger*.

THE LIFE CYCLE OF A CHRISTMAS ROSE FLOWER

Fig. 1 shows six characteristic stages in the life cycle of a typical Christmas rose flower. In the population we used in our experiments, the sepals were mostly pure white in the bud (**Fig. 1A**) and at anthesis (**Fig. 1B, C**) with only an occasional flush of pink on the outside. The proterogynous flowers first passed through their female phase (**Fig. 1B**) during which the stigmata were receptive, the immature anthers were arranged in a ring at the base of the cluster of carpels, and the nectaries were erect (i.e. parallel to the carpels). The male phase (**Fig. 1C**) began with the elongation of the filaments, while the nectaries moved into the plain of the sepals; it ended with abortion of both the anthers and the nectaries. After pollination, the sepals first turned yellowish-greenish (**Fig. 1D**), then bright green (**Fig. 1E**), and maintained this color until shortly before seed shedding (**Fig. 1F**).

Depending on temperature, snow cover and, possibly, genetic background, the developmental sequence shown in **Fig. 1** may start at any time, from late November to mid April. The seeds generally mature by May, or maybe June at the highest altitudes (around 1500 m). The developmental stages mentioned in this article could thus not be defined on an absolute time scale. Instead, we resorted to changes in the overall appearance of the flower and to a number of morphological parameters, such as the lengths and weights of the sepals and fruit clusters and the length of the flower scape (Salopek-Sondi *et al*. 2002; Tarkowski *et al*. 2006).

How some of those, or related, parameters changed during flower development is illustrated in **Fig. 2**. When the bud appeared above ground, it was already 10-15 mm long. Its size thus increased only about twice before unfolding. Simultaneously, the flower scape grew at a much faster rate to attain its final length shortly after anthesis. The sepals appeared to elongate at bud opening, but there was little expansion afterwards. They spread gradually; the flowers thus tended to be bell-shaped, during the female phase, flattening to the shape of a saucer, or a flat bowl, as anthesis proceeded and fruit development was initiated.

For the purpose of the experiments described herein, we defined the female phase of Christmas rose flowers to last from bud opening to beginning anther elongation. This was assumed to include the period of stigma receptivity, as was explicitly shown to be the case in the related species, *H. foetidus* L. and *H. bocconei* Ten., under average field conditions (Vesprini and Pacini 2005). However, when insect visits were excluded (simulating extended periods of cold and/or rainy weather when insects cannot fly) the stigmata could remain receptive for weeks (always depending on ambient temperature – see Vesprini and Pacini 2005 for details), well into the period of pollen shedding. Under these circumstances, autonomous self-pollination afforded 0.5-34% (depending on the population investigated) of the fruit-set observed in neighboring insect-exposed plants (Vesprini and Pacini 2000; Herrera *et al*. 2001). Christmas rose flowers were definitely cross-pollinated by bees and bumble bees, even on mild January days, if any. On the other hand, in springs following long and cold winters, some of the flowers emerging from the melting snow-cover bore ripening fruit. This was likely a consequence of autonomous self-pollination, because apomixis has so far not been observed in *Helleborus* (Vesprini and Pacini 2000).

THE STRUCTURAL AND FUNCTIONAL METAMORPHOSIS OF THE SEPALS

Following anthesis, the sepals lose their tender softness to become firm and more leaf-like in appearance, while their overall weight increases by 10-20%. This is mainly due to expansion of the preexisting cells and, even more so, of the intercellular spaces (Salopek-Sondi *et al*. 2002). The interior of a sepal thus takes on the appearance of the spongy mesophyll in a typical leaf, while the cells of the epidermis develop thickened outer walls.

In the perianth at anthesis, only the guard cells of the stomates contain chloroplasts. When the anthers abscise, the leucoplasts in the mesophyll-like tissue of the sepals develop a network of thylakoids and gradually attain the ultrastructure of photosynthetically active chloroplasts. The amount of photosynthetic pigments accumulating in the perianth appears to be under environmental and genetic control. In the material used in our experiments, chlorophyll, β-carotene, and xanthophyll levels increased from barely measurable concentrations to 350, 10, and 65 µg/g fresh weight (**Fig. 3**), which corresponded to one-third to one-fourth (chlorophylls and xanthophylls) to one-fifth (β-carotene) of the values found in mature leaves (Salopek-Sondi *et al*. 2000, 2002). These relationships were confirmed by Aschan *et al*. (2005), who likewise noticed variability depending on the origin of the plant material. The latter authors also studied *Helleborus viridis* L., which has a light-green perianth at flowering time, and found post-anthesis chlorophyll levels (expressed per leaf area) of about one-half the values measured in mature leaves.

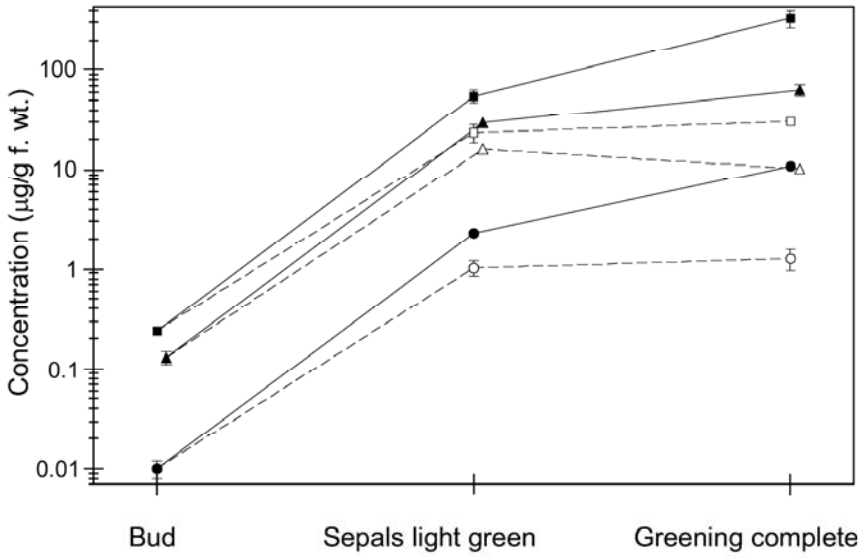

Fig. 3 Concentration dynamics of chlorophylls (squares), β-carotene (circles), and xanthophylls (triangles) during the life cycle of the Christmas rose perianth. Black symbols refer to intact flowers with developing fruit, plain symbols to flowers which were depistillated before anthesis, when the bud was about 25 mm long. Pigments were identified and quantified by HPLC. The values presented are arithmetic means of three independent analyses ± standard deviations.

Photosynthetic activity in green Christmas rose sepals was confirmed by cytochemical studies (photooxidation of 3,3'-diaminobenzidine) which specifically detect photosystem I (Salopek-Sondi *et al*. 2000). For photosystem II, the light-harvesting pigment-protein complex was isolated and identified immunochemically. Comparison of the amounts of this protein in green sepals and in mature leaves afforded about the same ratios as for chlorophyll levels (Salopek-Sondi *et al*. 2000).

The determination of photosynthetic capacities gave similar results. Under high-light conditions (quantitative parameters not available), oxygen production per gram fresh weight in green sepals was about 25% of that in mature leaves (Salopek-Sondi *et al*. 2000). Aschan and Pfanz (2003) determined the photon flux density optimal for photosynthesis in the sepals (600 µmol/m^2/s) and, under these conditions, measured electron transport rates of about 60% of those in mature leaves. These values are at the upper end of the range found for other flowers with chlorophyll-containing perianths. For instance, the inner tepals of snow-drops (*Galanthus nivalis* L.), which bear intensely green marks, afforded photosynthesis rates around 25% and electron transport rates around 60% of those in mature leaves (Aschan and Pfanz 2006). Comparable photosynthesis rates were measured for the green spots on the white perigon of *Leucojum vernum* L. (Prebeg *et al*. 1999). Young inflorescences of the terrestrial orchid *Spiranthes cernua* (L.) Rich. accomplished one-third of the leaf photosynthesis (Antlfinger and Wendel 1997), while the CO_2 fixation rate in the sepals of *Helleborus viridis* L. (Aschan *et al*. 2005) was 20-50% (depending on the CO_2-concentration), and that of green *Cymbidium* flowers was 10% (Dueker and Arditti 1968) of the values measured in the respective leaves.

IS PHOTOSYNTHETIC ACTIVITY IN CHRISTMAS ROSE SEPALS REQUIRED FOR NORMAL SEED DEVELOPMENT?

It has been postulated many times that floral photosynthesis can provide a significant part of the carbon needed for reproduction (Bazzaz *et al*. 1979; Reekie and Bazzaz 1987; Aschan and Pfanz 2003; Aschan *et al*. 2005; Aschan and Pfanz 2006), but rigorous proof of this plausible hypothesis is notoriously difficult. Circumstantial evidence is, however, quite persuasive.

In fact, before anthesis, the perianth of most flowering plants contains chlorophyll and performs photosynthesis; well-studied examples include the buds of 'Valencia' orange (Vu *et al*. 1985) and *Lilium* sp. (Clement *et al*. 1997a, 1997b). At anthesis, even the medullar cell layers of brightly colored petals may contain chlorophyll, which is masked by anthocyanins accumulated in the epidermis, as in the corolla of *Petunia hybrida* Vilm. (Weiss *et al*. 1988, 1990), or by light-reflecting intercellular spaces, as in the sepals of white carnations (Vainstein and Sharon 1993). In all these examples, however, the showy parts of the perianth abscise, following pollination. The assimilates they form may thus, at the best, help to cover the energy needs of the flower.

That the assimilates formed in the Christmas rose perianth are used in seed filling is suggested by the fact that most fruit weight is gained after the photosynthetic capacity in the sepals is fully established (Tarkowski *et al*. 2006). Also, the life-cycle of the flowers is almost complementary to that of the leaves. They survive normal winters, but are often pressed to the ground by snow and covered with debris, and thus no longer fully operative during anthesis. They will then die back around the time when fruit development is initiated. The new generation of leaves starts appearing a few weeks later and is not always fully expanded, at seed maturity (Werner and Ebel 1994). The exact timing depends on the weather. In an exceptionally warm and rainless spring, the overwintering leaves wilted at early anthesis, and replacement appeared well after fruit ripening (unpublished observations). Apart from the stores in the roots, the green perianth thus represented the only source of assimilates. The fact that viable seeds were nevertheless produced, strongly suggests that the fruit-bearing flower *can* be self-sufficient with respect to its carbon needs. There have so far been few attempts at establishing if this is *necessarily* so, even when assimilating leaves are present in the same plant. Working with *Helleborus foetidus* L. flowers (borne in a cluster on top of a leafy stem), Herrera (2005) found an approximately 10% decrease in final seed weight (but no effect on seed number) when he removed the sepals right after pollination. This may be significant in statistical terms, but not in the sense of proving a *preponderant* role for the green sepals in seed filling. Repeating the same experiment in *H. niger* did so far not produce easily interpretable results: almost no seed set in one year (Salopek-Sondi *et al*. 2002), but no difference to the controls in a second run (unpublished results), possibly because leaves were absent in the first, and present in the second experiment. We thus believe that the green sepals are the closest and most reliable, but not necessarily the only, source of assimilates for the developing Christmas rose seeds. This flexibility should be a competitive advantage for a species exposed, during fruit set, to the stressful, ever changing, weather conditions of a South-European winter and early spring.

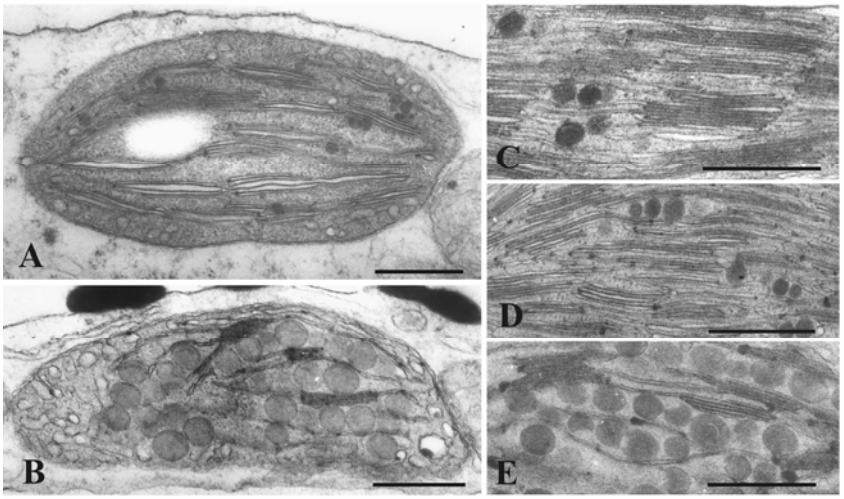

Fig. 4 Ultrastructure of chloroplasts in the post-anthesis perianth of *Helleborus niger* L. presented for a normal, fruit bearing flower (**A**), a flower de-pistillated at the bud stage (**B**), a depistillated flower treated with 1mM benzyladenine (**C**), a depistillated flower treated with 1 mM GA_3 (**D**), a depistillated flower treated with 1 mM indole-3-acetic acid (**E**). The growth regulators were applied in lanolin. The thylakoid system in chloroplasts from benzyladenine- and GA_3-treated depistillated flowers was even better developed than in green, fruit-bearing flowers. Untreated depistillated flowers contained plastids with only sporadic thylakoids and large plastoglobules, this general picture did not substantially change following auxin treatment. The length of the bars corresponds to 0.5 μm.

IS FLORAL PHOTOSYNTHESIS IN THE CHRISTMAS ROSE 'SINK- REGULATED'?

As early as 1868, Boussingault proposed that there is cross-talk between photosynthetically active and assimilate-requiring tissues. In a recent review on this subject, Paul and Foyer (2001) defined source-sink interactions as a highly integrated network, in which information on the carbon and nitrogen status of any plant tissue interacts with phytohormone-mediated pathways and redox signals. In fertilized Christmas rose flowers, the fruit are developing into an, increasingly demanding, 'sink', while 'source' tissues are simultaneously forming in the perianth. A plausible question to ask is thus: what happens to the sepals when the pistils are *not* pollinated?

Such flowers were occasionally found in natural Christmas rose populations following cold and rainy springs. They appeared to survive about as long as their fertilized neighbors, but the pistils remained at the size they had at anthesis and, more importantly, the sepals turned yellowish, sometimes mixed with a bit of green, but never became as brightly green as in seed-bearing flowers.

In an attempt to reproduce these effects under controlled conditions, we removed the pistils before bud-opening. These 'depistillated' flowers also survived about as long as neighboring, intact, flowers of the same age, but the sepals did not pass through a complete greening process (Salopek-Sondi *et al*. 2000). In quantitative terms, this is shown in **Fig. 3**. Chlorophyll, β-carotene, and xanthophyll levels were not affected to exactly the same extent, but, for all three pigment classes, the difference to the seed-bearing controls was obvious, in particular, when taking into account that the data are presented on a logarithmic scale. In addition to staying pale, depistillated flowers unfolded more slowly and eventually remained bell-shaped, with spreading angles as low as 60º (Salopek-Sondi *et al*. 2002). Also, the growth of the flower scape slowed down substantially, after pistil removal – in intact flowers of the same age, its elongation continued at a fast rate.

Only some of the other species with persisting, re-greening sepals were tested for survival of unpollinated or depistillated flowers. In the orchids belonging to this group (*Cleisostoma koordersii* Rolfe, *C. latifolium* Lindl., *Listera ovata* R. Br., *Dendrobium antennatum* Lindl., *Phalaenopsis violacea* Hort. ex H. Witte, *P. cornu-cervi* Blume & Rchb. f., *Promenaea* sp., *Epidendrum macrochilum* Hook.) unpollinated flowers aborted right after anthesis (van Doorn 1997). This was also reported to happen to the spathe of *Zantedeschia aethiopica* Spreng. when the spadix bearing the developing fruit was removed (Pais 1972).

CAN PLANT HORMONE TREATMENT SUBSTITUTE FOR THE ROLE OF DEVELOPING CHRISTMAS ROSE FRUIT?

As removal of the pistils at the bud stage redirects the life cycle of a Christmas rose flower, its normal development must be controlled by signals originating in the developing seeds. Fruit tissues are generally known as rich sources of plant hormones (Bearder 1980; van Staden 1983). We thus tested three groups of plant hormones which might be released by developing *Helleborus* fruit: cytokinins [N^6-benzyladenine (BA), *trans*-zeatin (Z), *trans*-zeatin riboside (ZR), dihydrozeatin (DZ), dihydrozeatin riboside (DZR), N^6-(Δ^2-isopentenyl)adenine (iP), N^6-(Δ^2-isopentenyl)adenine riboside (iPR)], gibberellins [GA_3, GA_4, GA_7], and auxins [indole-3-acetic acid (IAA), 4-chloroindole-3-acetic acid (4-Cl-IAA)]. They were applied in lanolin, in concentrations ranging from 0.01 to 10 mmol/kg (0.01 to 10 mM), to the inner surfaces of the sepals of freshly opened flowers, which had been depistillated at the bud stage. When fruit-bearing flowers of the same age were two to three weeks before seed ripening, the hormone-treated depistillated flowers were monitored for 1) sepal greening, 2) sepal spreading, and 3) elongation of the flower scape.

The effects on perianth greening were obvious. In general appearance, the sepals of depistillated flowers treated with 1 mM BA, Z and ZR were barely distinguishable from those of fruit-bearing flowers of the same age. The gibberellins tested were comparably effective – the auxins were not. Quantitative evaluation of pigment levels was not attempted, in this case, because the lanolin used as a rain-proof hormone carrier would have interfered with the extraction and chromatographic separation. Ultrastructural studies (Salopek-Sondi *et al*. 2002) revealed, however, that cytokinin and gibberellin treatment increased the number of plastids per cell and, at the same time, promoted their metamorphosis, from leucoplasts at anthesis, to chloroplasts with abundant grana thylakoids as indicators of strong photosynthetic activity (**Fig. 4**).

The effects on sepal spreading and scape elongation were less conspicuous because both processes were already well advanced (**Fig. 2**) when the sepals were wide enough apart to permit application of a hormone paste to their inner surfaces. Still, careful comparison of the spreading angles before and after hormone application strongly suggested concentration and structure-dependent cytokinin effects (for details see Salopek-Sondi *et al*. 2002). The ribosides tested (ZR, DZR, iPR) appeared to be more effective than the corresponding free bases. 0.1 mM ZR, 1 mM Z, and 10 mM iPR increased the spreading angle of depistillated flowers to about the same level as in fruit-bearing flowers which was, in this particular experiment, about 20º larger than in untreated depistillated controls. GA_3 (1 and 10 mM) had about the same effect. Auxins caused the tips of the sepals to close

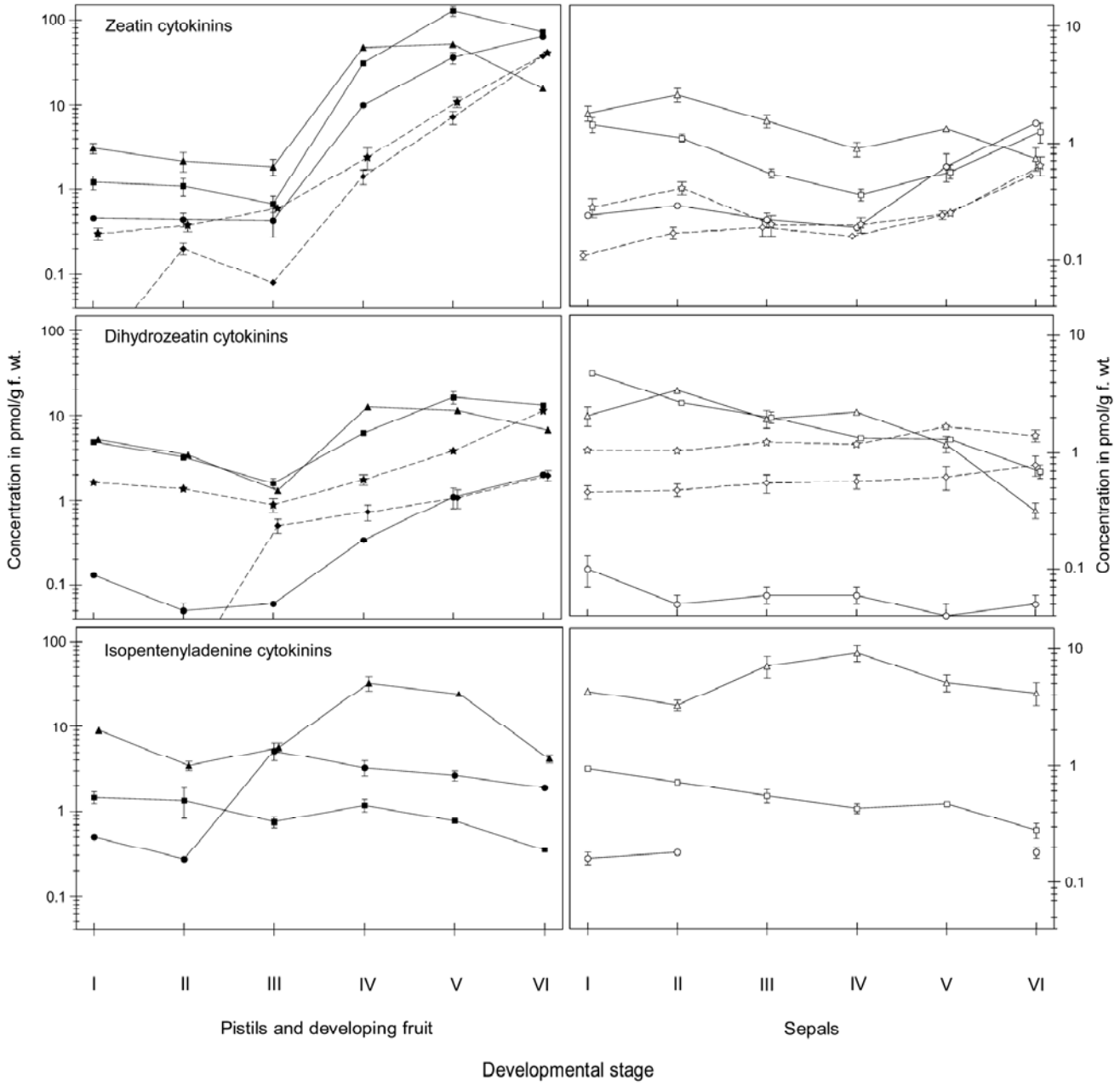

Fig. 5 Cytokinin dynamics during the life cycle of a Christmas rose flower. The developmental stages monitored were: I, anthesis female phase; II, anthesis, male phase; III, initial perianth greening; IV, advanced perianth greening; V, perianth greening complete; VI, two to three weeks before seed ripening. The data points shown are arithmetic means (n = 6–10) ± standard errors. They are presented by the following symbols: circles, free bases; squares, ribosides; triangles, riboside monophosphates; diamonds, *O*-glucosides; stars, riboside *O*-glucosides. The concentration of 9-glucosides was less than 0.06 pmol/g f. wt. except, in the fruit, for Z9G in stages IV – VI (0.21 ± 0.01, 0.76 ± 0.22 and 3.30 ± 0.92 pmol/g f. wt.) and DZ9G in stage VI (0.29 ± 0.04 pmol/g f. wt.). The iP levels in the sepals remained within the same general range, throughout the period monitored, but the results for stages III – V were not reproducible enough to be shown as definite data points.

by up to 20º, instead of spreading.

For elongation of the flower scape, only the effects of 10 mM GA_3 and 10 mM 4-Cl-IAA were statistically significant. In all other treatments, the response was obscured by sample variability, and more conclusive experiments are required. Auxin(s) and gibberellin(s) have been repeatedly implicated in the elongation of flower scapes (Kaldewey 1957; Hanks and Rees 1975; Izhaki *et al*. 1996). The role of gibberellins is corroborated by the fact that paclobutrazol (0.1 to 10 mM), as an inhibitor of gibberellin biosynthesis, inhibited scape elongation in Christmas rose flowers.

The range of hormone concentrations tested (0.01 to 10 mM) was much higher than plausible levels of endogenous growth regulators, but published data (Kaldewey 1957; Ross *et al*. 2000) indicate that this is necessary because hormones supplied in lanolin are taken up at very slow rates.

ENDOGENOUS PHYTOHORMONES

Cytokinins

As the application of cytokinins to the sepals of depistillated flowers mimicked some of the correlative signals released by the fruit developing in intact flowers, the concentration dynamics of endogenous cytokinins was of particular interest. After preliminary studies by HPLC (Salopek-Sondi *et al*. 2002), collaboration with the Plant Science Center in Umeå (Sweden) and the Laboratory of Growth Regulators in Olomouc (Czech Republic) permitted the use of mass spectroscopic techniques. Z, DZ, iP and their ribosides could be identified by full-scan mass spectra (as completely *O*-propionylated derivatives; Tarkowski *et al*. 2006). A protocol based on immunochromatography and liquid chromatography-mass spectrometry-single ion monitoring also re-

vealed the presence of the corresponding *O*-glucosides and riboside monophosphates, while 9-glucosides were only detected in some developmental stages, in very small amounts. The presence of *cis*-zeatin and aromatic cytokinins could not be conclusively shown. Cytokinin quantification was accomplished by isotope dilution, using deuterated internal standards. The results are summarized in **Fig. 5**.

The overall cytokinin levels in the carpels increased dramatically during early fruit development (while the sepals were going through the greening process). Riboside monophosphates (iPRMP, ZRMP, DZRMP) peaked first, followed by the ribosides ZR and DZR, while the free bases, Z and DHZ (but *not* iP), were most abundant in the most advanced stage analyzed (2–3 weeks before seed ripening). At anthesis, DZ-type cytokinins were slightly more abundant than their zeatin analogs, but, during fruit development, this proportion was inverted, with Z and derivatives attaining up to seven times higher concentrations. Zeatin type cytokinins were first isolated from immature corn seeds (Letham 1963, 1973) and appear to be abundant in developing fruits and seeds of other plant species as well (Letham 1978; Goodwin 1978). Recently studied examples include chickpea (Emery *et al*. 1998), white lupine (Emery *et al*. 2000), kiwi fruit (Lewis *et al*. 1996), and wheat (Lee *et al*. 1989; Banowetz *et al*. 1999).

The cytokinin dynamics shown in **Fig. 5** would be in accord with a biosynthetic pathway including iPRMP as a key intermediate (Sakakibara 2004). This implies the assumption that these cytokinins are produced *in situ*. In contrast to classical concepts (Letham 1994), recent research, such as hormone analyses in wheat ears cultured *in vitro* (Lee *et al*. 1989) and expression studies for genes of cytokinin biosynthesis (Miyawaki *et al*. 2004), has established fruit tissues as important sites of cytokinin formation. Also, it has long been assumed that only the free cytokinin bases are biologically active. This concept had to be abandoned when it was recently discovered that plants contain several cytokinin receptors, some of which also bind ribosides and riboside monophosphates (Maxwell and Kieber 2004; Spíchal *et al*. 2004; Yonekura-Sakakibara *et al*. 2004; Romanov *et al*. 2006). As long as the specific situation in Christmas rose flowers has not been clarified, it is difficult to relate the concentration dynamics of individual cytokinins to narrowly defined biochemical events. However, the overlap of cytokinin accumulation and more general patterns in seed and perianth development is at least very suggestive.

Two to three weeks before seed ripening, about 80% of the fruit cytokinins, but only 30% of the overall fruit weight, were in the seeds (Tarkowski *et al*. 2006). Their cytokinin concentrations must thus be even higher than represented by the data shown in **Fig. 5**. In other species, such as cereal grains (Morris 1997; Banowetz *et al*. 1999; Yang *et al*. 2002), white lupine (Emery *et al*. 2000) and chickpeas (Emery *et al*. 1998), cytokinin levels rise dramatically, after fertilization, to drop abruptly during the late ripening stage. This cytokinin pulse is usually correlated with cell division rates in the endosperm (Morris 1997; Yang *et al*. 2002), in accord with the role of these hormones in the cell cycle (Roef and van Onckelen 2004). It should, however, be remembered that, in these species, the embryo completes its morphogenetic phase (until the cotyledonary stage) at about the same time. In *Helleborus* seeds, both endosperm growth and embryo differentiation start around the time when the sepals begin turning green, but, as long as the seeds are connected to the mother plant, embryo development does not progress beyond an early cotyledonary stage (Niimi *et al*. 2006; Tarkowski *et al*. 2006). This rationalizes the constantly high cytokinin levels throughout fruit maturation, because experiments in *in vitro* cultures indicate that such high concentrations are required for embryo differentiation up to the cotyledonary stage (Nomura and Komamine 1985; Sagare *et al*. 2000; Tokuji and Kuriyama 2003). An alternative, or additional, explanation for the specific cytokinin dynamics of Christmas rose seeds would be a prolonged seed-filling period, as cytokinins increase the sink strength for assimilates (Paul and Foyer 2001).

Before perianth greening, cytokinin levels in the sepals and in the fruit were of the same order of magnitude. When overall fruit cytokinins increased precipitously, their pooled concentrations in the sepals did not pass through drastic changes. Only iPRMP peaked transiently, when perianth greening was initiated. The changes were less pronounced than in the developing fruit, but occurred in the same time frame. It is thus tempting to conclude that iPRMP is the cytokinin transported from the fruit to the perianth to induce the greening response, but additional confirmative evidence for such a translocation is clearly required. Indisputably, developing fruit contain sufficient amounts of cytokinins to supply the perianth.

To verify the effect of developing seeds on cytokinin levels in the sepals, depistillated and unpollinated flowers were analyzed (Tarkowski *et al*. 2006). The seedless flowers contained no riboside monophosphates, and the levels of most other cytokinins were also smaller than in the sepals of fruit-bearing flowers of the same physiological age (i.e. two to three weeks before seed ripening). The seedless flowers also weighed less and did not pass through a complete greening process. This indicates that cytokinins play a part in the normal life cycle of the perianth including the greening response following fertilization. A similar conclusion was reached for the spathe of *Zantedeschia aethiopica* Spreng. which also changes colors, from white to green, during fruit ripening. This did not occur (and the spathe died) when the developing fruit were removed, but their effect could be mimicked by applying a kinetin solution (Pais 1972). When these experiments were done, the analytical methods available were not yet sensitive enough to permit detailed insight into the cytokinin dynamics in the regreening spathe. The developing fruit were, however, shown to be a rich source of cytokinins of which 6-(*o*-hydroxybenzylamino)-9-β-D-ribofuranosylpurine (*o*-topolin riboside) and 6-(*o*-hydroxybenzylamino)-2-methylthio-9-β-D-ribofuranosylpurine were isolated and identified by chemical methods (Chaves das Neves and Pais 1980a, 1980b).

So far, the impact of cytokinins on the photosynthetic apparatus has mostly been investigated in model systems based on the prevention of chlorophyll loss as one of the symptoms of senescence (Richmond and Lang 1957; Medford *et al*. 1989; Gan and Amasino 1995), an analogy which now appears less straightforward than originally believed (Werner *et al*. 2001; Ananieva *et al*. 2004). The reverse process, cytokinin-mediated conversion of leucoplasts into chloroplasts (Parthier 1979) was, for instance, observed in a line of tobacco callus, which grew well without external cytokinin, but nevertheless required it for the formation of chloroplasts when the, normally dark-grown, tissue was transferred to the light (Stetler and Laetsch 1965). Greening processes of this kind are akin to what happens in the Christmas rose perianth, after fertilization, when the leucoplasts present at anthesis develop into chloroplasts (Salopek-Sondi *et al*. 2000, 2002).

Gibberellins

Gibberellins of seed origin are also likely to be involved in the coordination of fruit and perianth development, but their identification and quantification is so far not complete. Preliminary evidence by gas chromatography-mass spectrometry-selected ion monitoring suggests the presence of GA_1 and GA_4 in the seeds and, at least GA_1 (in much smaller amounts), in the sepals. Developing fruit thus appear to have the capacity to supply gibberellins to the perianth. More detailed studies are in progress.

THE CHRISTMAS ROSE AS A MODEL PLANT

The Christmas rose is well-suited for experimental work on flower biology. All the elements of their large flowers are accessible, surgical operations are easily performed, and a

single flower is, in many cases, sufficient for pigment, hormone, and related biochemical analyses. In particular, the fruit-bearing Christmas rose flower is a simple system for studying developmental aspects of source-sink interactions. The metamorphosis of the perianth to a photosynthetic organ is induced by signals released by the fruit developing in the same flower, and there is so far no evidence for interference by signals originating elsewhere in the plant. As soon as photosynthesis is initiated, the perianth becomes the closest source of assimilates for use in seed filling, but it is difficult to exclude any 'help' by the leaves and by mobilization of resources stored in the roots. Experiments with isolated seed-bearing flowers could clarify the situation.

Admittedly, the genetics of *H. niger* is by no means defined as completely as for more customary model plants, such as *Arabidopsis thaliana*. As far as the genes involved in flower development are concerned, we should, however, be able to learn from the records of plant breeders who have done at least 150 years of crossing and selecting, to bring trends such as flower size, shape and color, the length of the flower scape, and the timing of anthesis, in line with horticultural need and fashion (Mathew 1989; Rice and Strangman 1993). Understanding the signaling mechanisms involved in flower development may, in turn, pave the way for more targeted breeding strategies aimed at improvement of existing varieties. Other horticulturally important topics, which would profit from more detailed information on *H. niger* physiology, would be seed germination, which is still incompletely understood (Lockhart and Albrecht 1987; Niimi *et al*. 2006) and *in vitro* propagation (Seyring 2002).

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