Pollination Drop in *Juniperus communis*: Response to Deposited Material

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Background and Aims The pollination drop is a liquid secretion produced by the ovule and exposed outside the micropyle. In many gymnosperms, pollen lands on the surface of the pollination drop, rehydrates and enters the ovule as the drop retracts. The objective of this work was to study the formation of the pollination drop in *Juniperus communis*, its carbohydrate composition and the response to deposition of conspecific pollen, foreign pollen and other particulate material, in an attempt to clarify the mechanism of pollination drop retraction.

Methods Branches with female cones close to pollination drop secretion were collected. On the first day of pollination drop exposure, an eyelash mounted on a wooden stick with paraffin was used to collect pollen or silica gel particles, which were then deposited by contact with the drop. Volume changes in pollination drops were measured by using a stereomicroscope with a micrometer eyepiece 3 h after deposition. The volume of non-pollinated control drops was also recorded. On the first day of secretion, drops were also collected for sugar analysis by high-performance liquid chromatography.

Key Results The pollination drop persisted for about 12 d if not pollinated, and formed again after removal for up to four consecutive days. After pollination with viable conspecific pollen, the drop retracted quickly and did not form again. Partial withdrawal occurred after deposition of other biological and non-biological material. Fructose was the dominant sugar; glucose was also present but at a much lower percentage.

Conclusions Sugar analysis confirmed the general trend of fructose dominance in gymnosperm pollination drops. Complete pollination drop withdrawal appears to be triggered by a biochemical mechanism resulting from interaction between pollen and drop constituents. The results of particle deposition suggest the existence of a non-specific, particle-size-dependent mechanism that induces partial pollination drop withdrawal. These results suggest that the non-specific response may decrease the probability of pollen landing on the drop, reducing pollination efficiency.

Key words: *Juniperus communis*, female cone, gymnosperm reproduction, pollen, pollination drop withdrawal.

INTRODUCTION

The different landing sites for pollen in gymnosperms and angiosperms led Robert Brown to distinguish these land plant groups in the third decade of the 19th century (Maheshwari, 1960). Vaucher (1841) subsequently recognized the pollination drop as the landing site in many gymnosperms. Pollination drops appeared very early in the phylogeny of plants. According to Doyle (1945) and Rothwell (1977) pteridosperms and early gymnosperms already possessed a pollination drop and this mechanism of pollen capture was widespread during the Carboniferous (Taylor and Milway, 1979).

The pollination drop is a liquid secretion produced by the ovule and exposed outside the micropyle (Doyle, 1945). *Abies*, *Cedrus*, *Larix*, *Pseudotsuga* and *Tsuga* sp. (Pinaceae) have stigmatic micropyles and no pollination drop. In *Auracaria*, *Agathis*, *Tsuga dumosa* and *T. heterophylla* pollen lands on sites different from the micropyle (Singh, 1978; Owens et al., 1998). In other gymnosperms, pollen lands on the surface of the pollination drop or on a nearby cone surface and is then picked up by the pollination drop (Owens et al., 1998), rehydrates, and enters the ovule as the drop retracts. It is now known that pollination drops and cones enhance and facilitate pollen capture (Niklas, 1985; Greenwood, 1986; Buchmann et al., 1989; Owens et al., 1998; Roussy and Kevan, 2000).

The micropylar secretion contains dissolved substances, the composition of which was unclear at the beginning of the last century (Fujii, 1903; Tison, 1911). Subsequent studies found carbohydrates, amino acids and proteins. McWilliam (1958) found glucose, fructose and sucrose. Ziegler (1959) found several amino acids, peptides and organic acids. Sugars, and especially sucrose, glucose and fructose, are the more frequent substances identified in the pollination drop in more recent studies (Owens et al., 1987; Séridi-Benkaddour and Chesnoy, 1988; Gelbart and von Aderkas, 2002) that also revealed differences in the relative proportions of these substances in various groups of gymnosperms and angiosperms sensu lato. *Ephedra*, for instance, has a high proportion of sucrose and *Welwitschia* has a high percentage of fructose (Carafa et al., 1992). Acid phosphatase activity has been demonstrated in pollination drops of *Welwitschia* (Carafa et al., 1992). Proteins have been found in the micropylar drop of *Taxus* and Douglas fir and have been linked to pollen selection and development (O’Leary et al., 2004; Poulis et al., 2005).
Pollination drop secretion has been studied in several species of gymnosperms (Owens et al., 1987; Takaso and Owens, 1995a, b; Takaso and Owens, 1996). Ultrastructural modifications of nucellar apex cells during pollination drop secretion were reviewed by Takaso and Owens (1995a) and demonstrate that these cells are responsible for pollination drop secretion.

There is more uncertainty regarding the mechanism of pollination drop withdrawal. According to Tomlinson et al. (1997), pollination drop withdrawal is not determined by evaporation or mechanical stimulation but pollen is the sole stimulus for drop withdrawal. Biochemical and ecological aspects of pollination drops are largely unknown, as observed by Chesnoy (1993) in her review of the subject.

The aim of the present experimental study was to obtain information about pollination drop composition, production and withdrawal in Juniperus communis (Cupressaceae), and to determine if physical contact of non-pollen substances can influence pollination drop withdrawal. This work is part of a research programme on the reproductive biology of Mediterranean junipers (Mugnaini et al., 2004) that are of ecological importance because of their capacity to colonize highly stressed environments (Falinski, 1980; Piotto and Di Noi, 2003).

MATERIALS AND METHODS

Plants of Juniperus communis L. used for this research grew close to Greve in Chianti (Florence province), 43°37′68″ N 11°17′62″ E at an altitude of 184 m. Material was collected from 20 plants of approximately the same age and height (about 2 m).

Pollination drop volume and pollination experiments

Branches with cones just about to secrete the pollination drop were collected in the study area the evening before the experiments and kept in the laboratory. When drops appeared, sprigs 3–5 cm long with one/three female cones were inserted in vials containing water, and maintained overnight at 15 ± 1 °C and 52 ± 5 % relative humidity. Next morning sprigs bearing female cones without pollination drop were discarded. Female cones were only used for experiments on the first day of pollination drop exposure.

A human eyelash mounted on a wooden stick with paraffin was used to collect pollen and deposit it by contacting the pollination drop. Contact with the human eyelash alone did not stimulate withdrawal of the pollination drop.

The following were used for the pollination trials: (1) viable conspecific pollen collected soon after pollen sac opening; (2) dead conspecific pollen, killed by heat (105 °C for 12 h); (3) particles of silica gel in two size ranges (10–15, and 63–200 μm); (4) pollen of Pyrus communis; and (5) pollen of Pinus canariensis. The last two treatments were chosen to test if completely different kinds of pollen have a similar effect. Only one treatment was applied to the cones of each sprig. Viability of juniper pollen for treatments (1) and (2) was evaluated by means of fluorochromatic reaction (FCR) as indicated by Nepi et al. (2005).

A stereomicroscope with micrometer eyepiece was used to measure volume changes in ‘undisturbed’ pollination drops for a period of 48 h. Volume changes 3 h after pollination trials were observed via the same method. Pollination drop diameter, assumed spherical, was measured and its volume calculated with the formula $\frac{4}{3}\pi r^3$ where $r$ was the radius of the pollination drop. Only that part of the pollination drop protruding from the micropyle was observed and measured.

Pollination drop volume at time zero (i.e. just before deposition of pollen or particulate) was compared with that 3 h later by the Wilcoxon test for paired data. Although the time required for complete withdrawal of the droplet after pollination with conspecific pollen was 1–2 h, pollination drop volume was monitored for longer to be certain of any volume fluctuations or new secretion. Three hours after deposition of material on the pollination drop, it was possible to distinguish three different responses: complete withdrawal when pollination drop volume dropped to 0; partial withdrawal when pollination drop volume decreased significantly but did not drop to zero; and no withdrawal when there was not a statistically significant variation in pollination drop volume or a volume increase. The statistical significance of different behaviour of the pollination drop in response to the different treatments was evaluated by the chi square test applied to a $7 \times 3$ (treatments $\times$ response categories) contingency table.

Pollination drop collection and analysis

For chemical analysis, droplets were removed from sprigs, stored as described above, with a microcapillary tube (5 μL) on the first day of secretion. Samples were stored at −80 °C. The content of six capillaries, each containing 40–300 pollination drops, was analysed for sugar content by high-performance liquid chromatography (HPLC) as reported by Nepi et al. (2003). Before analysis, samples were thawed to ambient temperature and diluted 1:50 with distilled water. A 20-μL volume of sample and standard solution was injected into a Waters LC module 1 apparatus. Mobile phase was water (MilliQ, pH7). The flow rate was set at 0.5 mL min$^{-1}$ and column temperature at 85–90 °C. Sugars were separated in a Waters Sugar-Pack I (6.5–300 mm) column and identified with a refractive index detector (Waters 2410).

RESULTS

Pollination drop formation

The pollination period lasts about 1 month (Table 1). The three upper scales of the female cone open 1–2 d before pollination drop production and micropylar apertures are revealed. Pollination drop production starts in a few cones of each plant, reaching a peak when almost all cones of all plants in a study area have pollination drops. Pollination drop production in a single cone is not always a synchronous phenomenon.
Under laboratory conditions, ‘undisturbed’ pollination drops persisted for up to 12 d (Table 1). Average pollination drop diameter was 0.31 mm (Fig. 1), about 4–5 times larger than that of the micropyle (0.07 mm). Mean volume on the first day of emission was 0.02 mm$^3$ (range 0.002–0.122 mm$^3$). The volume of unpollinated pollination drops followed for 48 h fluctuated slightly in time under uniform laboratory conditions.

Because female cones have three ovules and the pollination drops are very close to each other, they sometimes merge spontaneously or in response to handling. When pollination drops were removed with a microcapillary tube, they formed again the next day or the day after. New pollination drops formed again up to four times; after that the ability to produce the drop was lost.

**Pollination drop composition**

Glucose, fructose and mannitol were detected. Fructose concentration was much higher (45.49 $\pm$ 0.59 mg mL$^{-1}$) than that of glucose (8.30 $\pm$ 0.35 mg mL$^{-1}$; Table 1). Mannitol was found in small amounts. Sucrose was not detected.

**Response of pollination drop to pollen or other powdery material**

When the pollination drop was touched with an eyelash free of pollen it did not retract or change in volume. Withdrawal occurred only when powdery material adhered to its surface (Fig. 2A–F). When pollen of *Juniperus communis* was applied, some grains went directly into the pollination drop but others remained on the surface, moving to the lowest point of the pollination drop along the surface. Pollen deposition caused evident pollination drop withdrawal in about 30 min.

All treatments were associated with a significant decrease in pollination drop volume except in the case of silica gel particles of 63–200 $\mu$m, which were associated with an increasing trend in pollination drop volume (Fig. 2D) and in the case of control pollination drops that did not alter in volume (Fig. 2G). The treatment that gave the most homogeneous response was that with viable pollen ($\chi^2 = 78.06$, d.f. = 12, $P < 0.001$). Complete pollination drop withdrawal was recorded in a significantly greater number of drops pollinated with viable conspecific pollen of *J. communis* (Fig. 3A). Partial withdrawal was characteristic of drops pollinated with non-viable pollen (Fig. 3B). Silica gel of 10–15 $\mu$m, *Pinus* and *Pyrus* pollen caused partial or total pollination drop withdrawal (Fig. 3C, E, F). A significantly higher number of non-withdrawals was recorded only in drops treated with silica gel of 60–200 $\mu$m and in control drops (Fig. 3D, G).

**DISCUSSION**

**Pollination drop production**

Gymnosperms show variations in the interval during which the pollination drop remains on the micropyle. Indeed, in genera such as *Juniperus* the pollination drop may persist for several weeks (Table 1); in other species it seems to be a cyclical phenomenon, i.e. secretion at night or in the early morning, when the atmosphere has high relative humidity, and evaporation or withdrawal during the day, with the cycle repeating the next day (Singh, 1978; Owens *et al.*, 1980). The cycles may be one or many, according to species. For instance, *Cupressus sempervirens* has five cycles, irrespective of whether or not pollination occurs (our unpublished data). Most authors (McWilliam,

![Fig. 1. A female cone of *Juniperus communis* with three micropiles, each one with a pollination drop. Scale bar = 0.3 mm.](https://academic.oup.com/aob/article-abstract/100/7/1475/216376/1477)
1958; Barner and Christiansen, 1960; Owens et al., 1980) have related these cycles to mechanisms that indicate a tree’s water status as the driving force behind drop production. By contrast, O’Leary and von Aderkas (2006) found that post-pollination drop production of hybrid larch is not regulated by a tree’s overall water status, but is under the control of localized structures such as the cone or ovule.

Unpollinated drops remain exposed for up to 2 weeks in *Taxus brevifolia* (Anderson and Owens, 2000) with the maximum volume observed in the early morning. Tomlinson et al. (1997) found that unpollinated drops of *Phyllocladus* (Phyllocladaceae) in field conditions evaporated slowly and could persist for over 1 h, but they persisted for several days in closed containers, becoming increasingly viscous, as suggested by stretching their surface with a

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**Fig. 2.** Effects of deposition of different powdery material on volume of pollination drop of *Juniperus communis*: (A) viable pollen; (B) non-viable pollen; (C) silica gel, 10–15 μm; (D) silica gel, 63–200 μm; (E) *Pinus* pollen; (F) *Pyrus* pollen; (G) control. Closed symbols indicate pollination drop volume before treatment, open symbols volume 3 h after treatment. Each point on the x-axis corresponds to a pollination drop. The table summarizes the statistical significance of each treatment according to the Wilcoxon test for paired data: n, number of trials; Z, statistics of the test; P, probability.
needle. This phenomenon was not observed in the present laboratory experiment with persistent unpollinated drops of *J. communis* that were exposed for almost 12 d. It therefore seems that in *J. communis*, an equilibrium establishes after some time between secretion and evaporation of the pollination drop. The lack of increase in viscosity suggests the existence of an osmotic mechanism of regulation affecting drop concentration. It is worth remembering that the laboratory conditions under which these observations were made may favour water availability, thus lowering solute concentrations of the pollination drop.

In *Juniperus communis*, when the pollination drop was removed, up to four cycles of re-secretion were possible, after which the pollination drop was not produced again. Similar observations were reported by Tomlinson et al. (1997) for *Phyllocladus* and by von Aderkas and Leary (1999) for Douglas fir and larches. It can be concluded that a limited number of secretion processes of the pollination drop can occur.

The chemical composition of the pollination drop

The chemical composition of the droplet and its concentration may be involved in the development of appropriate (conspecific) pollen. Sugars, amino acids and other substances and their relative abundance may create environments more or less favourable for pollen germination and tube growth. Thus, the pollination drop may exercise direct female selection of conspecific pollen and therefore have a prezygotic selective role (Gelbart and von Aderkas, 2002; Poulis et al., 2005).

Pollination drop sugar composition does not appear to be very different among the species studied thus far. Although sucrose is the preferred compound for carbon transfer in conifers (Gelbart and von Aderkas, 2002), fructose is the dominant sugar in *Pinus nigra*, *Pinus engelmannii* (McWilliam, 1958), *Cephalotaxus drupacea*, *Thuya orientalis*, *Taxus baccata* (Séridi-Benkaddour and Chesnoy, 1988) and *Welwitschia mirabilis* (Carafa et al., 1992). The present study also confirmed this trend in *Juniperus*. Only two exceptions seem to exist: *Picea engelmannii* has glucose as the dominant sugar in the pollination drop (Owens et al., 1998) and *Ephedra helvetica* has sucrose (Ziegler, 1959). The absence of sucrose or its subordination to glucose and fructose may be the effect of invertase activity in the pollination drop, as demonstrated in Douglas fir, in which two of the most abundant proteins in ovule secretions are invertases (Poulis et al., 2005).

*Pinus mugo* pollen cultivated in vitro shows preferential uptake of fructose with respect to other sugars (Nygaard,

![Fig. 3. Frequency histograms of the response of *Juniperus communis* pollination drop to deposition of different material (treatments): 1, complete withdrawal (pollination drop volume dropped to 0); 2, partial withdrawal (pollination drop volume decreased significantly but did not drop to zero); 3, no withdrawal (there was no significant variation in pollination drop volume or a volume increase). (A) Viable pollen; (B) non-viable pollen; (C) silica gel, 10–15 µm; (D) silica gel, 63–200 µm; (E) *Pinus* pollen; (F) *Pyrus* pollen; (G) control.]
1977). Fructose is presumably the ‘preferred’ sugar for development once pollen reaches the pollination drop.

Pollination drop withdrawal

The process of withdrawal has rarely been studied. It is evidently a quick process but it is not easy to discern what is responsible for this withdrawal and which cells collect/store the liquid. The first observations of withdrawal of the pollination drop after pollen deposition were made with cones of Pinus (Doyle and O’Leary, 1935) and subsequently with several other species such as Callitris, Chamaecyparis, Cryptomeria and Thuja (Tomlinson et al., 1997, and references therein).

According to Singh (1978) and references therein, pollination drop withdrawal takes from 10 min, as in Pinus, to much longer, as in Abrotaxis. These data cannot be taken alone: to understand the process it is necessary to know other features, such as pollination drop composition/viscosity, ovule morphology and climatic conditions at pollination. Pollination drop withdrawal seems hindered by high relative humidity, as demonstrated by the experiments of Lill and Sweet (1977) and Tomlinson (1994).

Different species of gymnosperms respond differently to pollen deposition: for some of them, pollen arrival is the stimulus for pollination drop withdrawal; for others pollination drop reduction simply occurs by evaporation, as for example in Podocarpaceae (Tomlinson et al., 1991, 1997). The present results with J. communis suggest that withdrawal of the pollination drop occurs in two phases that may occur simultaneously or sequentially.

A first phase involves partial withdrawal and occurs whenever particles are deposited on the pollination drop surface, irrespective of their biological or non-biological nature. In every case there is a significant variation in drop volume, suggesting that at least at first, the mechanism of withdrawal is linked to a non-specific physical stimulus. It also seems that in this first phase the diameter of particles is important. Inorganic particles 10–15 μm in diameter induce partial withdrawal of the drop whereas larger ones (63–200 μm) have no effect. As the diameter of the micropylar canal of J. communis is about 70 μm, the absence of the effect of the larger particles (63–200 μm) may be due to their inability to enter the micropylar canal. On the basis of the current experiments, the conclusion of Tomlinson et al. (1997) that inorganic material did not stimulate pollination drop withdrawal may have depended on the large size of the particles used in their experiments (glass Balloti spheres, diameter about 75 μm).

A statistically significant increasing trend of pollination drop volume after deposition of silica gel in the size range 63–200 μm emerges from the present observations. However, it is necessary to consider that initial pollination drop volumes in this treatment were much smaller than average, suggesting that these cones were in the early stages of secretion and would naturally tend to increase in volume. Differences in the stage of secretion may explain the high variability of pollination drop volume observed at any moment.

The fact that stimulation of the drop by simple temporary contact (such as touching with an eyelash or a dissecting needle) does not cause withdrawal had already been observed by Tomlinson et al. (1997) and could indicate the need for prolonged mechanical stimulation to determine withdrawal.

In the case of pollination with conspecific (juniper pollen) or heterospecific pollen (Pyrus and Pinus pollen), partial withdrawal may be caused by pollen rehydration. This is particularly true for juniper pollen, which has very thick hygroscopic intine (Pacini et al., 1999; Nepi et al., 2005), and for pine pollen, which, although not having thick intine relative to pollen size (Pacini et al., 1999), increases greatly in volume on hydration. In the case of silica gel, water molecules adhere to the surface but there is no ‘sponge effect’ with increase in particle volume. This phase therefore seems to be independent of the hygroscopic capacity of the particles that fall on the pollination drop.

The second phase determines total withdrawal and is only completed if viable conspecific pollen lands on the drop; this suggests the existence of a biochemical–molecular mechanism that can be triggered only by biological particles, such as pollen, and that does not respond to the stimulus of non-viable pollen, heterospecific pollen or abiological particles. Only the interaction between living conspecific pollen and the complex biochemical environment of the pollination drop leads to total drop withdrawal and subsequent pollen development. For some angiosperm species several types of metabolites (heterogeneous proteins—most of which are enzymes—sugars, amino acids, phenolics, polyamines) are known to be released by pollen and to interact with the stigma, style and ovary tissues, favouring pollen germination and tube growth (Shivanna, 2003, and references therein). What kind of metabolites are released by gymnosperm pollen and how they can interact with the pollination drop is not known.

CONCLUSIONS

The present results show that the pollination drop of Juniperus communis responds differently to stimuli determined by deposition of different particles. The ability to recognize viable and non-viable pollen is striking. Besides clarifying the mechanisms of pollination in J. communis, the results also show a certain non-specificity in the pollination biology of this species. The micropylar pollination of gymnosperms, especially J. communis, is less selective than the stigma pollination of angiosperms. The latter have a series of tissues and organs (stigma, style, ovary) that function as a ‘filter’ for pollen selection (Wilhelmi and Preuss, 1999; Shivanna, 2003). We have demonstrated that non-viable conspecific pollen, heterospecific pollen and also abiotic particles of an appropriate size induce partial withdrawal of the pollination drop of J. communis. Partial withdrawal of the pollination drop leads to a decrease in the surface exposed for pollen capture, reducing the probability of successful pollination. Thus, deposition of non-viable pollen or inorganic particles may reduce pollination efficiency in Juniperus by preventing viable pollen from reaching the surface of the nucellus.
and may have major ecological implications. We intend to test this hypothesis on natural populations of \textit{Juniperus} growing near sources of airborne dust, such as quarries, cement plants and roads. According to the laboratory experiments, the reproductive efficiency (i.e. the number and quality of seeds produced) of these populations should be lower than that of control populations growing far from sources of dust.

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LITERATURE CITED


