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Defoliation of common ragweed by *Ophraella communa* beetle does not affect pollen allergenicity in controlled conditions

S. T. E. LOMMEN[1,*], S. CIAPPETTA[2,*], A. GHIANI[2], R. ASERO[3], R. GENTILI[2], H. MÜLLER-SCHÄRER[1], & S. CITTERIO[2]

[1]*Departement de Biologie/Ecologie & Evolution, Université de Fribourg, Switzerland;* [2]*Department of Earth and Environmental Sciences, Università di Milano-Bicocca, Italy and* [3]*Ambulatorio di Allergologia, Clinica San Carlo, Italy*

Abstract

Ragweed allergy is one of the primary causes of seasonal allergies in Europe and its prevalence is expected to rise. The leaf beetle *Ophraella communa*, recently and accidentally established in N-Italy and S-Switzerland, represents a promising approach to control ragweed, but negative side effects should be excluded before its use. Since biotic and abiotic stresses are known to influence the allergenicity of pollen, we set out to assess the effect of sub-lethal defoliation by *O. communa* on the quantity and quality of ragweed pollen. Seventeen sister pairs (including six clones) of ragweed plants were grown in controlled conditions. One of each pair was exposed to *O. communa* as soon as the plant started to produce reproductive structures. After 10 weeks of exposure, plant traits were measured as a proxy for pollen quantity. Pollen quality was assessed by measuring its viability and allergenicity. Generally, plants produced very few male flowers and little amount of pollen. Damage by the beetle was severe with most of the leaf tissue removed, but no treatment effect was found on any of the quantitative and qualitative traits assessed. In conclusion, *O. communa* did not increase the amount or allergenicity of ragweed pollen grains in our experimental conditions.

Keywords: Ambrosia artemisiifolia, *common ragweed, pollen, allergenicity,* Ophraella communa, *biological control*

Introduction

Ambrosia artemisiifolia L. (common ragweed), a North America native plant, has been accidentally introduced to Europe where it has naturalized since the 19th century. It represents an increasingly serious threat to both environment and human health. The high ability of adaptation, reproduction, and dispersal make this plant a good competitor in disturbed areas affecting the existing plant diversity (Fenesi & Botta-Dukát 2012). *Ambrosia artemisiifolia* also has become a major weed in European agriculture, especially in spring-sown crops such as sunflower, maize, sugar beet, and soybean (Komives et al. 2006). In addition, *A. artemiisifolia* produces large quantities of highly allergenic pollen representing one of the main causes of pollinosis in many regions of the word (Smith et al. 2013). In Europe, given the high prevalence of sensitized people, social and economic impacts are significant (D'Amato et al. 2015). For instance, the annual health costs related to ragweed allergy have been estimated at € 110 million in Hungary, € 88 million in Austria (Gerber et al. 2011, and references therein) and more than € 1.7 million in a 90 km^2 area in North Italy (http://www.aslmi1.mi.it/), areas all highly invaded by *A. artemiisifolia*.

Common ragweed is continuing to expand across Europe, and future changes in climate and land use are expected to facilitate further spread to currently unsuitable areas (Essl et al. 2015). These changes can also augment the production of pollen (Ziska & Caulfield 2000; Singer et al. 2005). Hamaoui-Laguel et al. (2015) predicted a fourfold increase in airborne concentration of common ragweed pollen in Europe by 2050. This great increase in pollen concentration in the atmosphere along with the presence of pollutants, which can increase the allergenic potential of pollen (Zhao et al. 2016), constitutes a further alarming threat to human health.

Current management of *A. artemisiifolia* is mainly based on the use of broad-spectrum herbicides and mowing (Bohren et al. 2006; Patracchini et al. 2011), the latter of which has been enforcedly adopted by several local health authorities and municipalities to reduce pollen production, but effects are limited so far (Müller-Schärer et al. 2014). Another promising approach is classical biological control, where

Correspondence: S. Citterio, Dipartimento di Science Ambientali, Università di Milano-Bicocca, Piazza della Scienza 1, Milano 20126, Italy. Tel: +39 02 64482934. Fax: +39 02 64482996. Email: sandra.citterio@unimib.it
*These authors are contributed equally to this work.

natural enemies from the native range are introduced to control the plant in the invaded range (Müller-Schärer & Schaffner 2008). This method has proven to be permanent, environmentally friendly and a cost-effective control of several invasive plants (Seastedt 2015). In 2013, the leaf beetle *Ophraella communa* LaSage (Coleoptera: Chrysomelidae), already used as a biocontrol agent of ragweed in China (Zhou et al. 2011) and also effective against ragweed in Australia (Palmer et al. 2010), was unexpectedly found to have established in Northwestern Italy and southern Switzerland (Boriani et al. 2013; Müller-Schärer et al. 2014). The insect preferentially feeds on *A. artemisiifolia*, and severe defoliation can result in a reduction of flower and seed production, or can even kill the plant before flowering (Zhou et al. 2014). During the summer 2013 and 2014, concentrations of airborne ragweed pollen were significantly lower near the center of the colonized Italian area than what would be expected based on meteorological data of the region (Bonini et al. 2015). This strongly indicates a huge effect of the insect, and a direct benefit for human health. Nevertheless, before deciding on actively spreading this insect for ragweed control, potential concomitant negative impacts for human health, agriculture and the environment need to be carefully studied. Regarding human health, it is yet unclear how sublethal damage by *O. communa* will affect the quantity and quality of ragweed pollen produced. Non-lethal attack by aphids under controlled conditions reduced not only the quantity but also the viability and protein quantity of *A. artemisiifolia* pollen (Basky & Magyar 2009). However, a body of literature shows opposite effects, with several types of biotic and abiotic environmental stress increasing the severity and frequency of respiratory allergic diseases (Singer et al. 2005; Smith et al. 2013; Sinha et al. 2014; Zhao et al. 2015). Consequently, it cannot be excluded that non-lethal attack levels by *O. communa* may result in more aggressive (more allergenic) pollen.

In this paper, we report results of a laboratory experiment investigating the impact of defoliation by *O. communa* during flowering time on the quantity, viability, and allergenicity of pollen produced by common ragweed plants.

Materials and methods

Plant material

Both plants grown from seeds of single mother plants and clonal plants were used to test the effect of *O. communa* on ragweed. Seeds were collected from a ragweed population grown in the Botticino extraction basin (Brescia, Italy), an area not invaded by *O. communa*, in October 2009, and stored in paper bags at room temperature. In Spring 2015 they were cold-stratified in the dark at 4°C for 3 months, and then planted in a tray. Single seedlings of 20 mm long were transferred to plastic pots (14 cm diameter × 14 cm height) and grown in controlled condition (temperature: 25°C; 10 h dark/14 h light, 150 μmol $m^{-2}s^{-1}$; humidity: 65%).

Some of the obtained plants were vegetatively propagated to produce clonal individuals. To this purpose plant shoots were cut into nodal segments with one leaf and one lateral bud. The nodal segments were then cultivated in tubes containing 5 mL of MS medium supplied with Gambourg (B5) vitamins and supplemented with 4,5 μM 6-benzylaminopurine (BAP) to induce shoots regeneration. After 30 days of cultivation, microshoots > 2–3 cm with three or more leaves were excised and cultured on MS medium plus Vitamin B5 containing 0,5 μM indole-3-acetic acid (IAA) for rooting and growth. Finally, rooted plants were transplanted to pots and growth along with the plant from seeds in controlled conditions as described above.

Ophraella communa

Egg batches and males of *Ophraella communa* were collected from *A. artemisiifolia* in Magnagno, ca. 40 km from Milano, on two occasions in July 2015. They were kept in aerated pots, provided with ample fresh leaves of *A. artemisiifolia* in the same room as the plants for a maximum of 10 days.

Experimental procedure

Plants were weekly checked for the presence of floral buds. Once the first buds appeared in July 2015, a total of 17 pairs of plants were formed, by choosing 11 pairs of sister plants (all from different mother plants) and 6 pairs of relative clones (from 6 individual plants) that were as uniformly in size as possible. Of each pair, one was randomly assigned to the beetle treatment, and the other served as control. Individual plants were subjected to their assigned treatment when their floral buds appeared, and all treatments started within a three-week period. The maximum height and width of the plants were measured as an indication of size. Plants were then caged with partially transparent insect-free white tissue, from the pot until just below the first inflorescence, where the tissue was carefully attached to a sponge that surrounded the stem of the plant to protect it (Figure 1(A)). Plants assigned to the *Ophraella* treatment received a centrifuge tube near their stem before the tissue was closed, and which contained six male beetles and three unhatched egg batches each containing a minimum of 10 eggs on pieces of leaves,

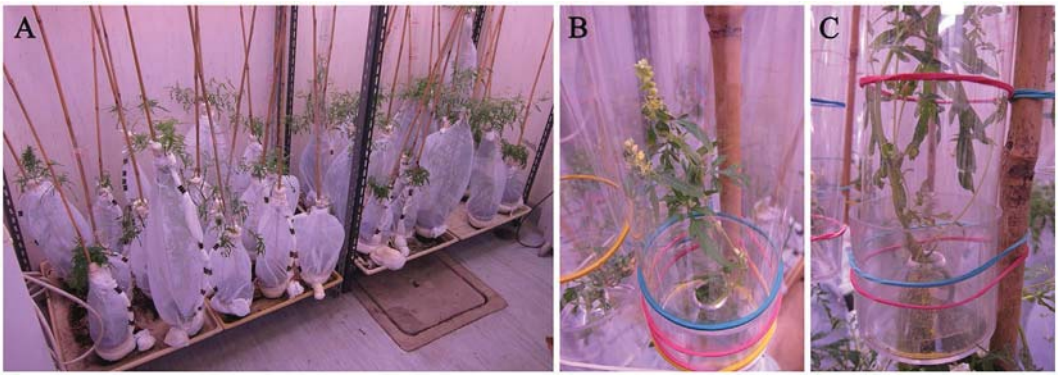

Figure 1. (A) Control and exposed plants caged with partially transparent insect-free white tissue. (B, C) Male inflorescences covered with transparent collectors (modified ARACON system) used to recover mature pollen.

collected 1–10 days before. In two cases where no beetles or larvae were seen after a week, the same amount of beetles was added again. In addition, we applied a cutting treatment to the uncaged leaves of all *Ophraella*-treated plants to simulate *Ophraella* feeding of those leaves that could not be accessed by *Ophraella*. Three weeks after the start of the treatment, 90% of the uncaged leaf tissue was cut manually in such a manner that only the veins remained, and this was repeated biweekly. When the caged part of the plant had been completely defoliated by the beetles (usually after four weeks), all remaining beetles were removed by an exhauster. Control plants were caged similarly but did not receive any beetles nor manual cutting. Mature pollen of each plant was recovered in transparent collectors, by covering 1–3 male inflorescences with a modified ARACON system (Kanter et al. 2013; Figure 1(B)) until 10 weeks after the start of the treatments.

Plant traits

We collected data on plant performance that are known to be well related with the amount of pollen produced (Fumanal et al. 2007; Šauliene et al. 2012). Plant height (cm), measured from the ground to the maximum growing point of the main branch, and lateral spread (cm), measured as the maximum diameter of the plant, were recorded just before the cage was installed and after 10 weeks of treatment. In addition, the following plant reproductive traits were recorded per plant: the number of racemes (the spikes with male flower heads), male flower heads, and female flowers, as well as the weight of the pollen collected.

Pollen viability

Membrane integrity and viability of pollen grains were estimated using the fluorescein diacetate (FDA) method (Heslop-Harrison & Heslop-Harrison 1970). This is based on the incubation of 0.1 mg of pollen with 1 ml of Mannitolo–FDA solution (0.3 M Mannitol and 0.01 mg mL^{-1} FDA) for 15 min in darkness at room temperature. The percentage of viable grains was estimated for single raceme by counting in a Bürker chamber using a standard fluorescence microscope (400× magnification) equipped with epi-illumination (Axioplan, Zeiss, Germany), 100 W halogen bulb, band pass 450–490 nm (blue) excitation filter, 510 nm chromatic bean splitter, and 520 nm long-pass filter. A pollen grain was considered as viable, when it emitted green fluorescence under blue excitation. Three independent experiments for each raceme were performed.

Pollen allergenicity

Slot blot technique was applied to assess the whole allergenicity of pollen collected from the different racemes. Soluble protein extracts were prepared according to Aina et al. (2010). Equal volumes of these extracts were bound to nitrocellulose membrane and first stained with Ponceau S staining solution [0.1% (w/v) Ponceau S in 5% (v/v) acetic acid] to assess the amount of proteins loaded in each well. Membranes were then used to evaluate the immunoreactivity of the different pollen extracts to a pool of sera from ragweed allergic patients, previously selected (Asero et al. 2014). Image analysis was applied to quantify reactivity signals. The integrated optical density (IOD) of immunoreactive spots with respect to the IOD of standard (Allergon®) was measured. At least three different samples for each racemes were analyzed.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism software for Windows (version 4.0

Table I. Mean ± standard error (SE) of the principal traits measured in beetle exposed and not-exposed (control) plants at the end of the experiment. No effect of the *Ophraella* treatment was found on any of the plant traits measured (ANOVA and Tukey's test or Kruskal–Wallis $p > 0.05$).

Plant traits	*N*	Control (mean ± SE)	*N*	Exposed (mean ± SE)
Plant height (cm)	17	42.71 ± 4.44	14	45.29 ± 3.55
Plant width (cm)	17	30.35 ± 4.26	14	26.50 ± 2.41
No of female flowers per plant	17	697.71 ± 237.29	14	219.93 ± 44.26
No of male racemes per plant	9	1.44 ± 0.13	8	1.75 ± 0.31
No of male heads per plant	9	32.75 ± 10.86	8	28.00 ± 8.85
Pollen weight per plant (mg)	6	2.68 ± 1.35	5	1.22 ± 0.28

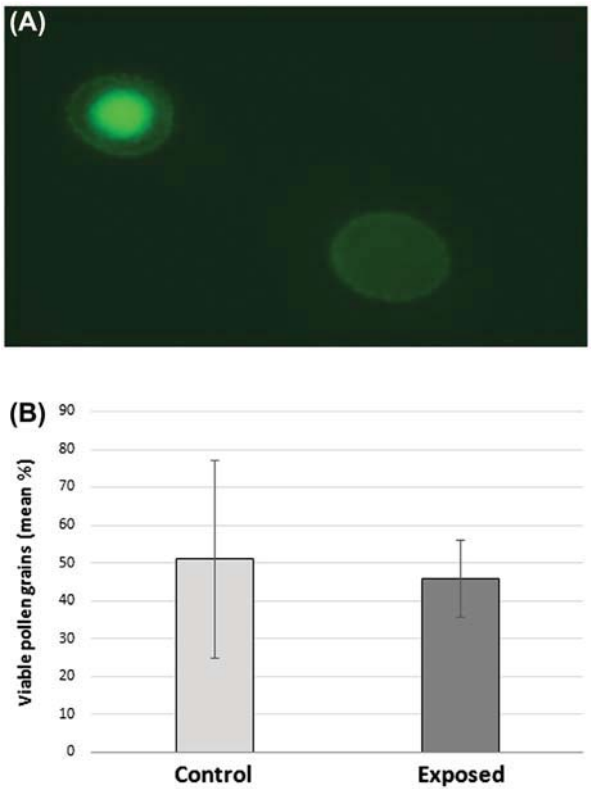

Figure 2. Viability of mature ragweed pollen released from flowers. (A) Staining with FDA: viable pollen grains show bright fluorescence; (B) mean percentage of viable pollen grains in control and *O. communa* exposed plants. No statistical difference between control and exposed plants was found, according to the Kruskal-Wallis non-parametric procedure ($p > 0.05$).

GraphPad Software Inc., San Diego CA): ANOVA and Tukey test were applied to the data when normality and homogeneity of variance were satisfied. Data not conforming to the assumptions (pollen viability, no of female flowers and racemes and pollen weight per plant) were log-transformed or analyzed by Kruskal-Wallis non-parametric procedure.

Results

Ragweed clones and plants from seeds were exposed to the beetle since the very beginning of their flower development for 10 weeks, whereas a sister plant/clone remained untreated. After this period, plant traits as a proxy for pollen production, along with the pollen viability and allergenicity were measured.

Ophraella feeding both by adults and larvae caused complete defoliation in the treatment cages and all but three plants (all from the *Ophraella* treatment) survived. In addition, we observed some damage by thrips on dried leaves on most of the treated and control plants. Remarkably, all plants produced predominantly female flowers, and only few male flowers. Half of all plants produced racemes, and sufficient amounts of pollen for quantitative analyses were collected from 6 controls (including two clones) and 5 *Ophraella*-treated plants (including 1 clone identical to one of the two control clones) before the end of the experiment. No effect of the *Ophraella* treatment was found on any of the plant traits measured at the end of the experiment (Table I). Also the clone pairs did not show any statistical difference ($p > 0.05$). Pollen viability determined by FDA staining (Figure 2(A)) was about 50% in both exposed and control plants (Figure 2(B)), and no effect of the beetle was found.

Total pollen allergenicity was assessed by slot blot technique. Figure 3(A) shows a representative membrane after immunodetection with a pool of sera from selected ragweed allergic patients. Image analysis was applied to quantify immunochemical signals: the IOD of immunoreactive spots with respect to the IOD of standard (sample IOD/standard IOD) was measured. On average, the reactivity signal of pollen samples from plants exposed to *O. communa* ranged from 0.97 to 1.04 whereas that of control plants ranged from 1.00 to 1.04 (relative units; Figure 3(B)). The mean values between treated and control plants were not statistically different ($p > 0.05$), indicating no effect of *O. communa*.

Discussion

Pollen allergenicity is widely recognized as a major determinant of health effects for sensitized patients, in addition to temporal and spatial allergen exposure (Cecchi et al. 2010). The allergenic potency of pollen, which is species specific, can be modulated by environmental conditions; many biotic and abiotic environmental stresses such as micro-organism infections (Won Jung et al. 2003), increased

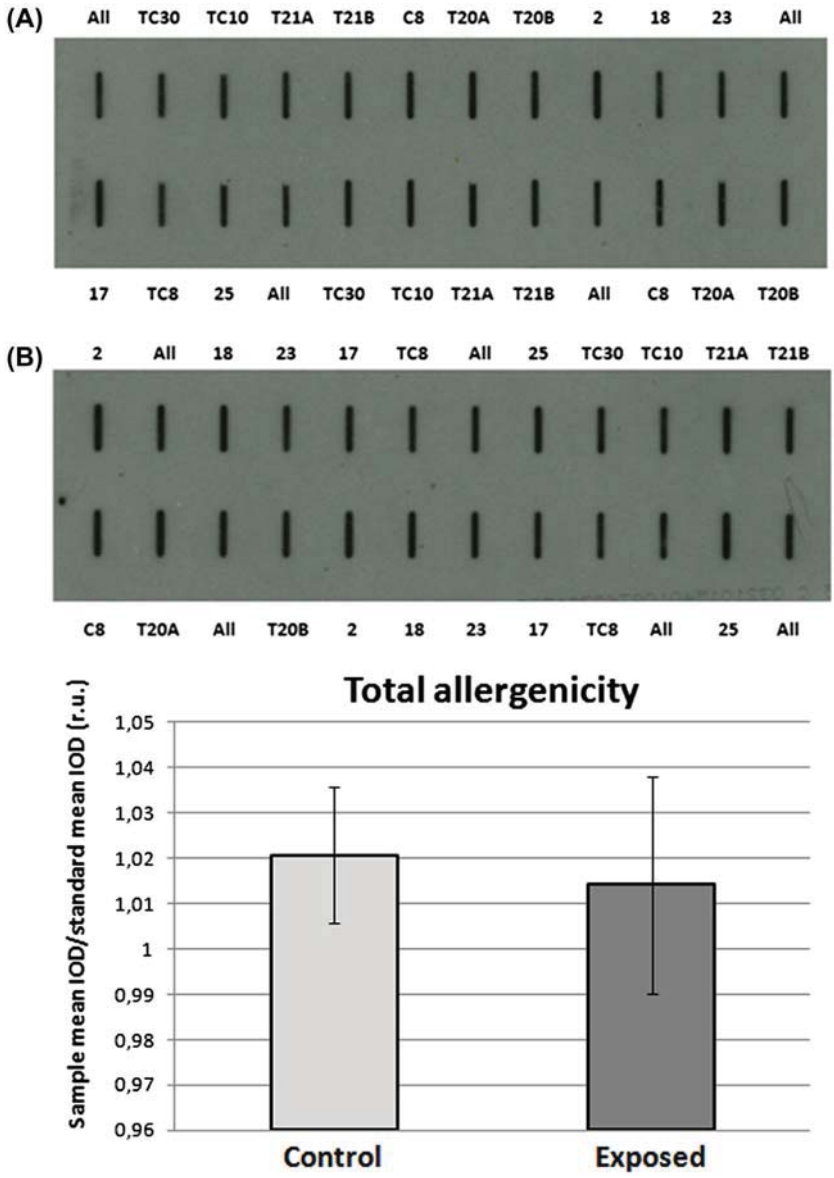

Figure 3. Total allergenicity of pollen samples from single racemes assessed with slot blotting. (A) Representative slot blot membrane probed with a pool of selected patient sera; All: internal standard (commercial pollen from Allergome). (B) Assessment of total pollen allergenicity through image analysis: the IOD of immunoreactive sposts with respect to the IOD of the standard (field sample IOD / standard IOD), was measured. The results reported are the mean (± standard deviation) of three independent experiments. ANOVA and Tukey test, $p > 0.05$ were performed; r.u.: relative units.

atmospheric greenhouse gasses, temperature, and air pollution (Shea et al. 2008; Smith et al. 2013), can in fact increase pollen allergenicity and consequently the severity and frequency of respiratory allergic diseases (D'Amato et al. 2015).

Pathogen attacks and insect feeding, in particular, trigger the expression of plant defensive proteins that exert direct effects on the antagonist or play a protective role for plant organs. Some of these proteins are allergens, which are accumulated in plant tissues. Examples are LTP allergens, which are proven to be transcriptionally activated by pathogen infection in *Capsicum annuum* tissues, and some classes of pathogenesis-related proteins (PRPs; Datta & Muthukrishnan 1999; Hoffmann-Sommergruber et al. 2000; Sinha et al. 2014). Thus, although the recent and accidental introduction of *O. communa* to Northern Italy represents a great opportunity to control *A. artemisiifolia,* the attacks by this insect also represents stress, and may therefore increase the amount or allergenicity of ragweed pollen in surviving plants. This may be problematic when these insects are used for biological control of the plants canceling its positive biocontrol effect on human health.

In our experiment, attack by *O. communa* and additional manual defoliation during flowering did

not alter the amount or allergenic potential of pollen from ragweed plants. Unfortunately no data concerning the effect of insect feeding or mechanical defoliation on fruit and pollen allergenicity have been carried out to date. Very few data are also available from experiments with viruses. Welter et al. (2013), for instance, found no effect of pepino mosaic virus infection on the allergenic potential of tomato fruits. This lack of effect is in line with our results and can be explained by taking into account the plant's ability and competence to respond to damage and the type of defense response. It is possible that *O. communa* induces the synthesis of non-allergenic defense compounds or activates a local, but not a systemic response and/or that the ability of ragweed defense response is restricted to specific development stages. A meta-analysis that summarized ontogenetic patterns in plant defense traits illustrated that, in the case of herbaceous plants, the ability to induce defenses (including secondary metabolites and proteins) after damage usually decreased with plant age. (Barton & Koricheva 2010). For instance, Quintero and Bowers (2011), showed that the ability of the annual plant *Plantago lanceolata* to induce chemical defenses against *Junonia coenia* was limited to juvenile, but not present in mature plant stages. Consequently, also common ragweed may preferentially synthetize defense proteins before flowering. In addition, it cannot be excluded that insect attack under field condition may induce a different plant reaction. Plants often have to cope with multiple attacks and belong to a population with genetically diverse individuals, which are not attacked at the same time and interact each other by exchanging signals. All these factors along with additional unpredictable surrounding environmental conditions modulate the complex regulation network of the plant's defense system influencing the expression of defense proteins including allergens. Furthermore, it should be taken into account that the effect of single biotic factors may be too weak to cause stable changes in the allergenicity of mature pollen and thus difficult to be observed by experiments in controlled conditions. Finally, the application of a cage around our plants and additional unintended infections may have affected our results. After caging, all the plants in the experiment grew slower, produced relatively large amounts of female flowers and very few male inflorescences, compared to the unused plants left uncaged (data not shown). Because stress due to the conditions, caging and unintended infections occurred in both exposed and control plants, it was still possible to assess the additional effect of the heavy stress of the defoliation treatment. In our experimental conditions, allergenicity of pollen was unaffected by the caging and infections, since there was no difference with unused plants left uncaged (ANOVA and Tukey test, $p > 0.05$) indicating that defoliation of mature *A. artemisiifolia* may indeed not affect allergenicity.

This study represents a first attempt to define the effect of *O. communa* on ragweed pollen amounts and allergenicity. More extended studies in controlled and field conditions are presently underway.

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