Pollen presentation, pollen-ovule ratios, and other reproductive traits in Mediterranean Legumes (Fam. Fabaceae - Subfam. Faboideae)

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Abstract. We studied the allocation of reproductive resources in 32 Mediterranean legume species. Correlations between main parameters were analysed at interspecific level. For each species we described the type of pollen presentation and examined its systematic significance. The high P/O values observed reflect low pollen transfer efficiency, typical of xenogamous species. Lowest P/Os were found in species with brush or explosive tripping mechanism, while highest ones were associated to primary pollen presentation. No trade-off was observed between P/O and the volume of a single pollen grain, nor between pollen number and pollen grain volume. Perennials showed higher P/Os compared to annuals, in accordance with the assumption that annual species are often self-compatible while most perennials are outcrossers. Fruit and seed set, evaluated in eight species under different experimental conditions, showed a close dependence on insects, and the existence of self-compatibility in three species (*Colutea arborescens*, *Coronilla emerus*, *Hedysarum coronarium*). In *Colutea arborescens* and *Coronilla emerus* pollen-pistil interaction and pre-zygotic barriers to selfing were investigated through stigma receptivity tests and in-vivo pollen germination trials. Results demonstrated the importance of the stigmatic cuticle in reducing levels of autogamy. Productivity tests confirmed the utility of P/O ratio as indirect measure of species' breeding system.

A great diversity of legumes belonging to the subfamily Faboideae is found in the Mediterranean region, where approximately 17 tribes, 79 genera and 1792 species are represented (Greuter et al. 1989), including lots of endemic and rare taxa. In spite of many taxonomical and agronomical studies, several investigations concern the reproductive biology of wild legume species (Karron 1987, Karoly 1992, Kaye 1999, Kittelson and Maron 2000, Suzuki 2000, Ben Brahim et al. 2001, Rymer et al. 2002, Pérez-Bañón et al. 2003, Galloni and Cristofolini 2003, Suzuki 2003) and the energetic investment in sexual reproduction (Cumaraswamy and Bawa 1988, Small 1988, Gallardo et al. 1994, Vonhof and Harder 1995, Ortega-Olivencia et al. 1997, Herrera 1999, Rodríguez-Riaño et al. 1999b, López et al. 2000, Herrera 2001) or both (Kudo and Molau 1999, Navarro 1999, López et al. 1999, Rodríguez-Riaño et al. 1999a, Galetto et al. 2000, Ehrlen and Van Groenendael 2001).

The subfamily Faboideae includes entomophilous species with different breeding systems: woody and perennials being generally

outcrossers, annuals and biannuals being self-compatible or predominantly autogamous (Arroyo 1981, but see Tandon et al. 2003).

Informations on breeding system can be obtained through indirect analysis of resource allocation in reproduction (Cruden 1977, 2000; Willson 1983) and with direct estimation of seed productivity and viability in natural populations.

Hermaphrodites allocate their reproductive resources to both male and female functions. Male reproductive function consists of pollen transfer to a compatible stigma and its success in ovule fertilization. Pollen germination and growth on the stigma depend upon many factors, including number and size of pollen grains. Female reproductive success is conditioned mainly by resource allocation to ovules, seeds and fruits, but also by pollen availability, fruit dispersal and resistance to predators (Charnov 1982). Charnov's sex allocation hypothesis for cosexual plants predicts an inverse relation between selfing rate and the allocation to male versus female function (Charlesworth and Charlesworth 1981, Charnov 1982, Queller 1984, Charlesworth and Charlesworth 1987, Lloyd 1987, Gallardo et al. 1994, Vonhof and Harder 1995). Sexual reproduction is in fact guaranteed in autogamous flowers, where pollination is not limited by external factors: less pollen grains per ovule, with higher probability of fertilization, are required by these species (Mione and Anderson 1992). Cruden (1977) proposed the ratio between pollen grains and ovules (P/O) to indirectly infer species' breeding system, reflecting the efficiency in pollen transfer, although the P/O ratio is not strictly equivalent to the relative male/female allocation (Charnov 1982, Queller 1984). Cruden and Miller-Ward (1981) reported the existence of a negative correlation (trade-off) between P/O and pollen grain size, due to the physiological requirements of pollen-pistil interaction after pollination. The sex allocation theory states that a negative correlation between P/O and pollen grain size is likely whenever both breeding system and cost per ovule remain constant (Gallardo et al. 1994). In species with similar breeding system, all factors reflecting a greater efficiency in pollination should influence negatively the P/O ratio (Cruden 2000). Even if Cruden's hypothesis is generally accepted, several authors highlight that other factors are needed for the interpretation of P/O (Preston 1986, Small 1988, Plitman and Levin 1990, Mione and Anderson 1992, Gallardo et al. 1994, López et al. 1999, Jürgens et al. 2002, Parachnowitsch and Elle 2004).

The relationship between entomophilous species and pollinators, and the degree of self-pollination, can be observed directly through field tests on fruit productivity in natural conditions and with artificial limitation to insect visits (Dafni 1992, Sage et al. 2005).

Self-fertilization in papilionoid flowers can be prevented either by physiological self-incompatibility or by dichogamy and mechanical barriers, commonly associated to tripping devices (Arroyo 1981, Yeo 1993, Pérez-Bañón et al. 2003, Suzuki 2003).

Multi-allelic self-incompatibility systems are widespread in Faboideae (Bubar 1958, Arroyo 1981, Heslop-Harrison and Heslop-Harrison 1982), with a higher frequency in woody groups compared to herbaceous ones.

Protandry has been reported to prevent autogamy in some perennial species (Couderc 1980, Juncosa and Webster 1989, Navarro 1999, Etcheverry 2001), although self-fertilization could happen through geitonogamy (Navarro 1999). A highly impermeable "stigmatic membrane" has been observed in different genera (Arroyo 1981, Heslop-Harrison and Heslop-Harrison 1983, Lord and Heslop-Harrison 1984, Shivanna and Owens 1989 cited by Yeo 1993, De Las Heras et al. 2001): it represents the cuticle of the stigmatic epidermis detached from the cells that formed it (Yeo 1993). This cuticle commonly breaks down spontaneously in autogamous species, while in entomophilous species with some degree of self-compatibility it retains the stigmatic fluid until ruptured by pollinators, reducing the risk of self-fertilization (Pazy 1984, Juncosa and Webster 1989,

Rodríguez-Riaño et al. 1999a, De Las Heras et al. 2001).

Our objective was to analyse the relationships between P/O ratio and: (i) breeding system, (ii) pollen presentation, (iii) taxonomic position, (iv) life form. Taxonomic significance of pollen release mechanism was also examined. We evaluated the reproductive success of eight legume species in natural populations under different field-experiments and performed in-vivo pollen germination tests in order to investigate pollen-pistil interactions and possible pre-zygotic self-incompatibility in two species.

Materials and methods

The studied species

We considered 32 legume wild species belonging to eight tribes of the subfamily Faboideae (see Appendix 1): 9 species representing 5 genera of tribe Cytiseae, 7 species representing 2 genera of tribe Vicieae, 4 species representing 3 genera of tribe Trifolieae, 4 species representing 4 genera of tribe Loteae, 3 species representing 2 genera of tribe Coronilleae, 2 species representing 2 genera of tribe Hedysareae, 2 species representing 2 genera of tribe Galegeae, and a single species of tribe Robinieae (the only one not autochthonous in the Mediterranean region). Life forms of examined species are detailed in Table 1. Vouchers are conserved in the University of Bologna Herbarium (BOLO).

Pollen release mechanism

The mechanism of pollen releasing has been described for each species, through direct observations in the field and on collected fresh sample flowers. Floral parts functioning in secondary pollen presentation (Yeo 1993) have been identified. Four main modalities (brush, piston, valvular and explosive) have been described for Fabaceae by previous authors (Polhill 1976, Arroyo 1981, Westerkamp 1996, López et al. 1999). Reviewed definitions of each mechanism are hereunder reported:

Brush mechanism: anthers dehisce before flower "opening" and pollen is presented to pollinators in a brush of stylar or stigmatic hairs (Lavin and Delgado 1990); more visits are necessary to empty the flower from pollen.

Piston or Pump mechanism: insect's pressure on the keel forces a pump-like action of stigma and stlyle which pushes a small amount of pollen to exit from an opening at keel tip: commonly flowers receive several visits.

Valvular mechanism: when the insect forces the wing-keel complex downwards, the reproductive column emerges from the keel and a little cloud of pollen is released. After insect's visit the flower parts return in the initial position, so that flowers are visited several times.

Explosive: the insect forces the wing-keel complex downwards, provoking a little explosion in which almost all pollen is released in a cloud. After the first successful visit flower parts become displaced: usually flowers are visited only once by legitimate pollinators.

Stigma receptivity and pollen-stigma interaction

Timing of stigma receptivity was determined following the procedure proposed by Dafni and Maués (1998) and Dafni et al. (2005), with Peroxtesmo esterase indicator paper liquid. Pistils were excised from flowers at three development stages: 1) flower buds with closed anthers; 2) flowers with closed corolla and anthers starting to dehisce; 3) flowers with opened standard and anthers dehiscent. We put stigmas on microscope slides in contact with a Peroxtesmo test paper dipped in a drop of distilled water. The appearance of blue color after several minutes indicates the presence of peroxidases. The test was repeated three times for each floral stage.

We checked the presence of the stigmatic cuticle in all species through staining experiments with Neutral Red and optical microscopy (Heslop-Harrison and Heslop-Harrison 1982) on stigmas excised after three pollination treatments: i) intact flower buds, ii) flower buds in which stigmas were touched with a paint-brush, iii) open mature flowers (probably visited). If a cuticle is present, intact stigma resists the penetration of dye Neutral Red; when it is ruptured, due to flower tripping or mechanical disturbance, the dye readily enters and occupies intercellular spaces: stigma surface appears then red colored. Treatments were performed on fresh sample flowers previously brought to the laboratory.

Table 1. Qualitative and quantitative characteristics of the flower in the studied species. LF = Life form: annual (A), woody perennial (W), herbaceous perennial (Hp). Pollen release mechanism: piston (P), valvular (V), explosive (E), brush (B); no secondary pollen presentation (I). Columns 5–9: P = number of pollen grains per flower, O = number of ovules per flower, P/O = pollen: ovule ratio, V = volume of single pollen grain, Vtot = total pollen volume per flower (P×V). Quantitative characters are expressed as mean value ± standard deviation

1	2	3	4	5	6	7	8	9	10
Species	Tribe	LF	Pollen release	P	O	P/O	V (μm^3)	V tot (mm^3)	Flower weight (mg)
Coronilla emerus	Coronilleae	W	P	51642±11311	14.0±1.0	3714±959	13340±2486	0.69±0.28	10.6±0.1
Coronilla varia	Coronilleae	Hp	P	23442±5183	15.3±2.0	1518±184	1320±225	0.03±0.01	4.6±0.0
Hippocrepis comosa	Coronilleae	Hp	P	31658±8177	10.7±2.3	3209±1670	5110±892	0.16±0.07	3.4±0.1
Cytisophyllm sessilifolium	Cytiseae	W	P	62418±13889	7.4±0.8	8623±2374	6838±727	0.43±0.14	4.5±0.1
Cytisus hirsutus	Cytiseae	W	V	75425±15605	12.7±0.6	5975±1328	5294±717	0.40±0.14	10.9±0.1
Cytisus scoparius	Cytiseae	W	E	55592±4375	14.7±2.0	3812±408	1380±147	0.077±0.014	19.2±1.5
Genista cilentina	Cytiseae	W	E	10020±2768	6.0±0.9	1689±507	6636±658	0.07±0.03	4.5±0.1
Genista januensis	Cytiseae	Hp	E	16921±3671	9.3±1.5	1803±112	11323±1971	0.19±0.08	1.5±0.1
Genista radiata	Cytiseae	W	V	24712±5337	4.4±0.7	5790±1413	7016±1381	0.17±0.07	8.3±0.1
Genista tinctoria	Cytiseae	Hp	E	28108±2245	10.0±0.0	2811±224	1535±184	0.043±0.009	4.6±0.1
Laburnum anagyroides	Cytiseae	W	P	74358±8050	15.1±1.4	12393±1342	6509±933	0.48±0.12	15.6±0.1
Spartium junceum	Cytiseae	W	E	63956±11957	6.0±0.0	4240±745	49519±8750	3.17±1.15	40.0±0.1
Astragalus glycyphyllos	Galegeae	Hp	V	19853±2776	22.0±2.0	911±183	13312±1509	0.26±0.07	5.2±0.1
Colutea arborescens	Galegeae	W	B	124500±4583	29.0±3.6	4326±402	23956±2926	2.98±0.47	28.9±0.1
Hedysarum coronarium	Hedysareae	Hp	I	75100±5597	3.7±0.3	20894±4164	5833±685	0.44±0.08	7.0±0.1
Onobrychis viciifolia	Hedysareae	Hp	I	23128±3287	1.0±0.0	23128±3287	1344±169	0.031±0.008	3.5±0.1
Anthyllis vulneraria	Loteae	Hp	P	3654±948	2.0±0.0	1827±474	3717±878	0.014±0.007	3.6±0.1
Dorycnium hirsutum	Loteae	Hp	P	45256±2044	32.7±2.0	1387±66	1460±225	0.061±0.013	14.9±0.0
Lotus corniculatus	Loteae	Hp	P	198500±13012	56.3±5.5	3558±553	1667±177	0.33±0.06	6.2±0.1
Securigera securidaca	Loteae	A	P	18899±1984	12.3±1.0	1539±225	922±201	0.017±0.006	2.7±0.1
Robinia pseudoacacia	Robinieae	W	B	23467±2238	15.3±2.1	1539±124	12595±1522	0.30±0.06	12.3±0.1
Medicago arborea	Trifolieae	W	E	5364±138	14.0±1.0	385±37	6796±1373	0.037±0.008	7.2±0.1
Ononis masquillirii	Trifolieae	W	P	53861±6174	6.0±0.0	8977±1029	832±92	0.05±0.01	7.3±0.1
Ononis natrix	Trifolieae	W	P	154517±5700	36.3±1.0	4253±172	5538±564	0.86±0.12	11.5±0.1
Trifolium alpinum	Trifolieae	Hp	V	9750±1819	2.0±0.0	4875±909	17426±2631	0.17±0.06	5.4±0.1
Lathyrus aphaca	Vicieae	A	B	4758±298	4.7±1.0	1033±166	4225±267	0.020±0.003	2.9±0.0
Lathyrus hirsutus	Vicieae	A	B	6667±1007	6.0±0.0	1111±168	3637±408	0.024±0.006	5.0±0.1
Lathyrus latifolius	Vicieae	Hp	B	45957±8634	19.0±1.7	2461±709	36245±6631	1.67±0.62	31.0±0.1

Lathyrus pannonicus	Vicieae	Hp	B	15700±2619	966±184	16.3±1.2	17220±2806	0.27±0.09	9.1±0.1
Vicia cracca	Vicieae	Hp	B	7467±2960	1183±590	6.7±1.0	2538±216	0.019±0.009	2.0±0.2
Vicia hybrida	Vicieae	A	B	10442±3244	1740±541	6.0±0.0	3325±538	0.035±0.016	10.1±0.1
Vicia sativa	Vicieae	A	B	6119±1417	511±120	12.0±1.0	2381±585	0.015±0.007	5.2±0.1

Reproductive success and pollen tube growth

The reproductive success was studied in the field on a sample of individual plants, randomly chosen in wild populations of eight selected perennials: seven woody species (*Cytisophyllum sessilifolium*, *Spartium junceum*, *Genista radiata*, *Genista cilentina*, *Colutea arborescens*, *Coronilla emerus*, *Cytisus scoparius*) and one herb (*Hedysarum coronarium*) (see Appendix 1 for details). Ovule, flower, fruit and seed production were monitored in: i) bagged racemes with unmanipulated flowers (spontaneous self-pollination) referred as "SSP", ii) bagged racemes with manipulated flowers (hand self-pollination) referred as "HSP", iii) control racemes (open to natural pollination) referred as "NP" treatment. Racemes were bagged with bride tulle (Dafni 1992, Sage et al. 2005) at beginning of anthesis to prevent insect visits and consequent entomophilous pollination. HSP and HCP treatments were performed using a paint-brush in order to break the stigmatic cuticle. The different experiments were done on the same individuals, the number of plants considered per population depending on species (see Appendix 1). Differences among treatments have been calculated for fruit and seed set (legumes/flowers and seeds/ovules). Sample size is indicated in Table 3.

In *Coronilla emerus* and *Colutea arborescens* we studied pollen-pistil interaction in sample flowers subjected to the following pollination treatments: i) "SSP" spontaneous self pollination (25 flowers in *Coronilla emerus* and 23 in *Colutea arborescens*), i) "HSP" hand-self pollination (23 flowers in *Coronilla emerus* and 21 in *Colutea arborescens*), iii) "HCP" hand-cross pollination (24 flowers in *Coronilla emerus* and 22 in *Colutea arborescens*), iv) "NP" natural pollination (20 flowers in *Coronilla emerus* and 22 in *Colutea arborescens*). In SSP, HSP and HCP treatments flower buds were bagged before anthesis. For both species we considered plants cultivated in the Botanic garden of Bologna: all flowers employed in the experiment were taken from a single individual, and a second plant was used as cross pollen donor.

The gynoecium was extracted from each flower at least 6 hours after the pollination treatment (gynoecia of *Colutea arborescens* were longitudinally cut in two parts before preparation), fixed in FPA solution (formalin 40%, concentrated propionic acid, 50% ethanol, 5:5:90 v/v/v) for 24 hours and stored in ethanol 70%. Excised gynoecia were

then softened in 8 N NaOH and stained with 0.1% aniline blue in potassium acetate until study. Samples were mounted on microscope slides with a drop of aniline blue (Dafni 1992, Dafni et al. 2005) and observed under fluorescence microscopy (Nikon ECLIPSE E600) to detect pollen grain germination on stigmas and pollen tubes growth in the styles. In both species the proportions of pistils with clearly germinated pollen grains (regardless of pollen tube length) and pistils with no germinated pollen grains were compared among pollination treatments (NP, SSP, HSP, HCP). Data were examined with a multiway contingency table and tested with a chi-square to assess whether differences among treatments were significant. Further examination with 2×3 tables was performed to establish which data sets contributed to the statistical significance.

Floral sex allocation

Sample flower buds were collected at early developing stage (with mature but still intact anthers) and transfered to the laboratory for the following analyses:

– determination of flower biomass (mean dry weight), as an indirect measure of flower size;
– removal of anthers' whorls (upper and lower) and estimation of mean number of pollen grains produced per flower (P);
– removal of the ovaries from the same flowers for counting of ovules (O);
– acetolysis of pollen samples (Erdtman 1969) to measure axes lenghts and calculate mean pollen grain volume (V).

Total pollen production per flower was calculated through estimation of mean number of pollen grains and total volume of pollen. Pollen grains were counted with a modified dilution method (Cruden 1977, Dafni 1992, Dafni et al. 2005, Sage et al. 2005) using three flowers per population. Anthers of different whorls were considered separately. Anthers were taken from fresh sample flower buds and squashed in 100 μl of ethanol/glycerol solution (3:1). For effective pollen removal, vials were put in ultrasonic horn for 2 minutes. A known volume (2 μl) of suspension was mounted with a drop of Calberla solution (Dafni 1992) immediately after 10 seconds of vial's sonication, to guarantee the homogeneity of suspension.

Grains were counted with help of an eyepiece grid and a manual counter (López et al. 1999). This number was multiplied by the dilution factor and then by the number of anthers to obtain the number of pollen grains for each anthers' whorl (basifixed and dorsifixed) and the total number of grains per flower. Pollen size was studied in acetolysed grains (Erdtman 1969) to avoid variability in size due to hydration stage. Acetolysed samples were prepared for light microscopy by mounting in glycerol jelly: 30 pollen grains per species were considered to estimate mean volume, based on the formula for an ellipsoid.

Data analysis

Prior to statistical analysis, data were tested for normality by means of Shapiro test. Non parametric tests were used in case of non-normal distributions after data transformation, or in case of low number of data. The analysis of correlation among floral sex allocation attributes was performed by using Pearson's correlation coefficient relative to means (Log-transformed data). To explore the influence of pollen grain number, pollen grain volume, and ovule number on flower size we used a multiple regression model. Subset analyses of floral sex allocation attributes (relative to tribe, pollen release mechanism and life form) were carried on considering groups of at least three data. Differences among groups were tested with Kruskal-Wallis ANOVA followed by separate Mann-Whitney U-tests (hereafter referred to as K-W and M-W, respectively).

Percentage data on fruit and seed set were angular transformed (Sokal and Rohlf 1995). Intraspecific variations among years and pollination treatments were tested with two-ways ANOVA. When three pollination treatments were considered, comparisons with pairwise t test were carried after Bonferroni adjustment to determine which ones differed significantly.

Statistical analyses were performed using R version 1.9.0 (R Development Core Team 2004).

Results

Pollen release mechanisms. Each of the pollen release mechanisms described for Fabaceae is

represented in the taxa considered (Table 1). Associations between tribe and mechanism (only searched for tribes with at least three species) were found only in Coronilleae, Loteae and Vicieae (piston mechanism characterizes the first two, brush mechanism the latter). Cytiseae and Trifolieae are the most heterogeneous tribes, with explosive, piston and valvular mechanism represented. All species, except *Hedysarum coronarium* and *Onobrychis viciifolia* (tribe Hedysareae) show secondary pollen presentation.

In species with secondary pollen presentation the anthers start to dehisce before flower opening, so that the stigma is usually embedded in the precociously released pollen mass.

Stigma receptivity and pollen-stigma interaction. In the majority of species (regardless of life form) stigma surface showed positive reaction to Peroxtesmo esterase indicator at early stage of floral development, when anthers were starting to dehisce (or even earlier in few cases) and petals were still closed. In some cases timing of stigma receptivity was delayed compared to anthers dehiscence, but male and female phases overlap at flower's opening (Table 2).

In all perennial species and one of the annuals (*Lathyrus hirsutus*) we detected the presence of a stigmatic cuticle: staining experiments with Neutral Red showed that stigmas previously touched with a tiny paint-brush and stigmas excised from mature open flowers were permeable to the dye, while stigmas excised from intact flower buds showed high impermeability, indicating the presence of a mechanical barrier. By contrast, in the other annual species considered, we detected dye penetration through stigmatic surface also in flower buds that were not previously manipulated, demonstrating the absence of any impermeable layer. Results concerning stigma receptivity and stigmatic cuticle are reported together (Table 2).

Reproductive success and pollen tube growth. Fruit and seed set (respectively expressed in percentage of legumes per flowers "L/F" and seeds per ovules "S/O") differ significantly among treatments in all species

(ANOVA, $p \leq 0.001$): in bagged racemes subjected to spontaneous self-pollination (SSP) fruit and seed production were null or almost null in all species, showing significant differences compared to controls (NP). For details concerning populations and years of study see Appendix 1. When available and not significantly different, we treated data from different years of study together to calculate overall mean and standard error.

In *Cytisophyllum sessilifolium* and *Spartium junceum* also manipulated flowers (HSP) produced no fruits. By contrast, hand self-pollination experiments gave rise to legume and seed production in *Colutea arborescens*, *Coronilla emerus* and *Hedysarum coronarium*, both fruit and seed set being significantly different from spontaneous self pollination treatment. In *Colutea arborescens* and *Coronilla emerus* the seed set in this test did not differ significantly from controls, while in *Hedysarum coronarium* S/O percentage was significantly lower compared to controls (Table 3).

The analysis of pistils submitted to different pollination trials showed significant differences among treatments both in *Colutea arborescens* and *Coronilla emerus* (*C. arborescens* $\chi^2 = 57.49$; *C. emerus* $\chi^2 = 40.92$; $p \leq 0.001$, df $= 3$). These statistical differences are mainly due to the fact that when visit by insects is excluded and stigmas are not manipulated (SSP), pollen germination appears to be generally precluded in both species (Table 4).

On the contrary, we observed germinated pollen grains and elongating pollen tubes in the styles of hand-self pollinated pistils, the proportion of germination being not significantly different either from hand-cross pollination treatment (HCP) or from controls (NP).

Sex allocation. Mean pollen grain number ranges from 3654 ± 948 in *Anthyllis vulneraria* to 198500 ± 13012 in *Lotus corniculatus*; mean ovule number ranges from 1.0 ± 0.0 in *Onobrychis viciifolia* to 56.3 ± 5.5 in *Lotus corniculatus*; mean P/O ranges from 385 ± 37 in

Table 2. Stigma receptivity and stigmatic cuticle tests: "+" = positive reaction, "−" = negative reaction. Stage 1 = flower bud with closed corolla, closed anthers; Stage 2 = flower with closed corolla, anthers dehiscent; Stage 3 = flower with open corolla, anthers dehiscent; Nt = not tested

Species	Stigma receptivity			Stigmatic cuticule
	Stage 1	Stage 2	Stage 3	
Coronilla emerus L.	−	−	+	+
Coronilla varia L.	−	−	+	+
Hippocrepis comosa L.	−	+	+	+
Cytisophyllum sessilifolium (L.) Lang	−	+	+	+
Cytisus hirsutus (L.) Link	−	+	+	+
Cytisus scoparius (L.) Link	−	+/−	+	+
Genista cilentina Valsecchi	+/−	+	+	Nt
Genista januensis Viv.	−	+	+	+
Genista radiata (L.) Scop	−	+	+	Nt
Genista tinctoria L.	−	+	+	+
Laburnum anagyroides Medicus	−	+/−	+	+
Spartium junceum L.	−	+	+	Nt
Astragalus glycyphyllos L.	−	+/−	+	+
Colutea arborescens L.	−	−	+	+
Hedysarum coronarium L.	−	−	+	+
Onobrychis viciifolia Scop.	−	−	+	+
Anthyllis vulneraria L.	−	−	+/−	+
Dorycnium hirsutum (L.) Ser.	−	−	+	+
Lotus corniculatus L.	−	+	+	+
Securigera securidaca (L.) Deg. & Dörfl.	−	−	+	+
Robinia pseudoacacia L.	−	+	+	+
Medicago arborea L.	+/−	+	+	+
Ononis masquillierii Bertol.	−	+	+	+
Ononis natrix L.	+/−	+	+	+
Trifolium alpinum L.	+	+	+	+
Lathyrus aphaca L.	+	+	+	−
Lathyrus hirsutus L.	−	+	+	+
Lathyrus latifolius L.	−	+/−	+	+
Lathyrus pannonicus L.	+/−	+	+	+
Vicia cracca L.	+	+	+	−
Vicia hybrida L.	+	+	+/−	−
Vicia sativa L. var. *angustifolia*	−	−	+/−	−

Medicago arborea to 23128 ± 3287 in *Onobrychis viciifolia*: in our sample there is a 60-fold range in P/Os (Table 1). According to Cruden (Cruden 1977, Dafni 1992) the studied species can be attributed to the categories of obliged or prevalent xenogamy.

Differences in mean values of P/O among tribes are statistically significant between Cytiseae and Vicieae (M-W U-test, p < 0.001) and between Cytiseae and Loteae (M-W U-test, p < 0.05) "Pump" and "brush" mechanisms manifest significantly different P/O mean values (M-W U-test, p < 0.01), but in the other cases no relationship has been found between P/O and pollen presentation (Fig. 1): anyhow, these results have to be taken

Table 3. Fruit and seed set in: control racemes (NP), spontaneous self-pollination treatment (SSP), hand self-pollination treatment (HSP)

Pollination treatment → SPECIES	Natural Pollination			Spontaneous Self-Pollination			Hand Self-Pollination			Differences between treatments: significance levels					
										NP - SSP		NP - HSP		HSP - SSP	
	F	L/F (%)	S/O (%)	F	L/F (%)	S/O (%)	F	L/F (%)	S/O (%)	L/F	S/O	L/F	S/O	L/F	S/O
Cytisus scoparius	1384	47.66±0.08	13.85±0.09	675	0.84±0.09	0.05±0.01	–	–	–	***	***	–	–	–	–
Genista radiata	2930	23.96±0.06	5.59±0.01	2455	0.023±0.004	0.00	–	–	–	***	***	–	–	–	–
Genista cilentina	1508	35.21±0.19	2.66±0.03	1496	0.08±0.01	0.010±0.002	–	–	–	***	***	–	–	–	–
Spartium junceum	757	6.03±0.08	3.43±0.07	885	0.00	0.00	25	0.00	0.00	***	***	–	–	–	–
Cytisophyllum sessilifolium	2047	6.46±0.06	0.68±0.01	1993	0.00	0.00	397	0.00	0.00	***	***	***	***	ns	ns
Colutea arborescens	198	30.41±0.58	13.18±0.29	128	0.12±0.12	0.02±0.02	104	19.50±1.01	6.86±0.48	***	***	ns	ns	**	**
Coronilla emerus	420	35.64±0.57	3.34±0.15	238	0.03±0.03	0.00	116	13.87±0.66	0.95±0.05	***	***	*	ns	***	*
Hedysarum coronarium	2200	37.89±0.44	18.89±0.18	2523	0.37±0.08	0.00	645	12.64±0.24	3.74±0.07	***	***	*	**	*	*

Abbreviations: F = sample size (number of flowers); L/F = Legumes/Flowers; S/O = Seeds/Ovules. Percentages are expressed in mean values±standard errors (back transformed). Differences between treatments are expressed with significance levels (ANOVA): ***p < 0.001; **p < 0.01; *p < 0.05; ns = not significant

Table 4. Pollen-pistil interaction in *Coronilla emerus* and *Colutea arborescens*. The total number of pistils in which pollen germination was observed and the total number of pistils with no germinated pollen grains are reported for each pollination treatment

Pollination treatments ↓	*Coronilla emerus*		*Colutea arborescens*	
	Pistils with germinated pollen grains	Pistils with NO germinated pollen grains	Pistils with germinated pollen grains	Pistils with NO germinated pollen grains
HSP	15	8	15	6
HCP	21	3	20	2
SSP	1	24	0	23
NP	15	5	21	1

Abbreviations: *HSP* = hand self-pollination, *HCP* = hand cross-pollination, *SSP* = spontaneous self-pollination, *NP* = natural pollination (control). Statistical differences between treatments: SSP vs. HSP (*C. arborescens* χ^2 = 24.9; *C. emerus* χ^2 = 20.2; $p \leq 0.001$, df = 1); SSP vs. NP (*C. arborescens* χ^2 = 41.2; *C. emerus* χ^2 = 24.5; $p \leq 0.001$, df = 1); SSP vs. HCP (*C. arborescens* χ^2 = 37.6; *C. emerus* χ^2 = 34.5; $p \leq 0.001$, df = 1); HSP vs. NP (*C. arborescens* χ^2 = 4.6; *C. emerus* χ^2 = 4.6; $p \leq 0.05$, df = 1). Differences not significant between treatments: HSP vs. HCP; NP vs. CP

cautiously, since the sample sizes are low for both valvular and explosive mechanisms.

Although not considered in the statistical analysis, *Hedysarum coronarium* and *Onobrychis viciifolia* (both belonging to tribe Hedysareae and both with primary pollen presentation) show the highest P/O values (20894 and 23128, respectively), more or less 5-fold higher than the overall average (Table 1).

For the 32 species examined, no significant correlation was found between pollen number and pollen grain volume ($p > 0.1$), P/O ratio and pollen grain volume ($p > 0.1$). Ovule number was positively correlated with both pollen number (r = 0.5, $p < 0.01$) and pollen biomass (Vtot) (r = 0.43, $p < 0.01$). Multiple regression analysis showed that flower size (dry weight) is significantly and positively affected by both pollen grain number and volume ($p < 0.01$ in both cases), but not by ovule number ($p > 0.1$). Greater P/O ratios were not associated with larger flowers ($p > 0.1$).

The number of pollen grains/flower and the pollen-ovule ratio vary with life form (χ^2 = 9.9, $p < 0.01$ and χ^2 = 8.0, $p < 0.05$, respectively): mean values of P and P/O are significantly higher in woody perennials compared to annual species (M-W U-test, $p < 0.01$

and $p < 0.05$, respectively). Between perennial herbs and annual species only the number of pollen grains differed significantly (M-W U-test, $p < 0.05$), while differences between
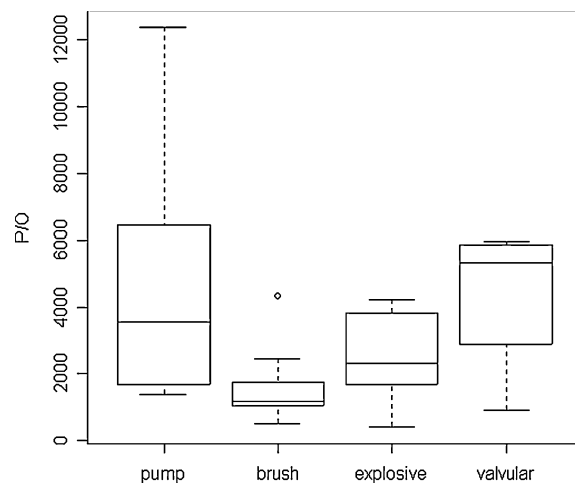

Fig. 1. Box-plot of P/O and pollen release mechanisms: median values are reported. Sample size: 6 Explosive, 11 Piston ("pump"), 9 Brush, 4 Valvular. The box itself contains the middle 50% of the data. The upper edge (hinge) of the box indicates the 75th percentile of the data set, and the lower hinge indicates the 25th percentile. The line in the box indicates the median value of the data

woody and herbaceous perennials were not statistically significant.

Discussion

The allocation of reproductive resources was studied in 32 Mediterranean legume species, including woody, herbaceous perennials, and annuals, in order to establish correlations among the main parameters at interspecific level. Correlations between P/O values, pollen presentation and life form were looked for, as a means to investigate differences in pollen transfer efficiency. The reproductive strategy of eight perennials was inferred from productivity tests; potential self-compatibility was demonstrated in two of them, and the presence of mechanical barriers to selfing (stigmatic cuticle) was also looked for.

Taxonomic significance of pollen release mechanisms. Pollen presentation is often connected with floral anatomical features. Therefore, there is in several cases a correspondence between pollen presentation and taxonomic groups, whose characterisation largely relies upon floral morphology. Nevertheless, the taxonomic significance of pollen release mechanisms must be treated with some caution, since morphological characters related to pollination strategies are adaptive characters, that may reflect convergent or parallel evolution rather than phylogenetic affinity.

In tribes Cytiseae, Vicieae, Trifolieae, Loteae, Coronilleae, Robinieae and Galegeae pollen is usually released while the flower is in the bud stage; corolla, style and filaments are the main structures involved in secondary pollen presentation. By contrast, in species belonging to tribe Hedysareae pollen is offered to pollinators directly by anthers, without involving other floral structures. Our observations on these tribes confirm literature data (Yeo 1993, Rodriguez-Riaño et al. 1999a).

Piston mechanism is characteristic of Coronilleae and Loteae, a functional homogeneity that supports the recent fusion of these tribes (Bisby et al. 2004).

Presence of a pollen brush deserves special attention. Lavin and Delgado (1990) recently re-defined the pollen brush more precisely as "a dense aggregation of erect trichomes emanating from the style and functioning in secondary pollen presentation", and distinguished it from ciliate and papillate stigmas or ciliate styles. Our observations are consistent with this definition and we also recommend to avoid vague terminology when using pollen brush as a taxonomic trait.

The presence of a pollen brush is a taxonomic marker of the tribe Vicieae. On the contrary, no such association has been observed in tribe Galegeae, for which the pollen brush is commonly used as a key character to distinguish the subtribe Coluteinae from others (Polhill 1981, Lavin and Delgado 1990). The reliability of this character, however, should be checked on a more complete set of species.

The type of pollen release has no systematic significance in Cytiseae and Trifolieae. These tribes include species with either piston, valvular or explosive mechanisms, or even species with more than a single type of pollen release: in *Cytisophyllum sessilifolium* pollen is released by piston mechanism until the last stages of anthesis, when the tripping becomes valvular (Polhill 1976).

Pollen-pistil interaction and reproductive strategies. Results concerning stigma receptivity and stigmatic cuticle emphasize that even if the stigma is potentially receptive at early stage of floral development, when it is covered by self-pollen grains, in perennial species habitually an impermeable stigmatic cuticle retains the stigmatic fluid, preventing pollen germination. When the cuticle is mechanically ruptured, such as at first pollinator visit, its function ceases, allowing hydration of the grains and pollen tubes to grow into the intercellular spaces of the stigma. The mechanism occurs independently from pollen presentation type and possible physiological incompatibility system (Arroyo 1981, Heslop-Harrison and Heslop-Harrison 1983, Yeo

1993, Rodríguez-Riaño et al. 1999a, Rodríguez-Riaño et al. 2001).

Our experiments on *Colutea arborescens* and *Coronilla emerus* show that, at bud stage, self-pollen germination is prevented when the stigmatic surface is intact, but the level of germinating pollen grains does not significantly differ in hand self or hand cross pollination trials, demonstrating that cuticle disruption may enhance both self- and cross-fertilization. Although not specifically tested, the existence of cryptic self-incompatibility is very probable in both cases, as the number of germinated cross-pollen on the stigmas was greater than that of self-pollen. Reduction of autogamy in these species is therefore accomplished at first by a morphological protandry made operational by the stigmatic cuticle. According to previous studies this behaviour seems to be widespread in the Faboideae (Heslop-Harrison and Heslop-Harrison 1983, Horovitz and Harding 1983, Pazy 1984, Asmussen 1993, Rodríguez-Riaño et al. 1999a, De las Heras et al. 2001).

Results concerning reproductive success in controlled conditions of eight perennial species demonstrate that in all cases insect visits are necessary for the production of seed. Even if self pollination does not occur in natural conditions, in at least three species we cannot exclude the existence of some degree of self-compatibility. In *Colutea arborescens*, *Coronilla emerus* and *Hedysarum coronarium*, hand self-pollination gave rise to seed production: seed set was lower but not significantly different from controls in the first two species, while in *Hedysarum coronarium* it was significantly reduced. These results indicate that a sporophytic incompatibility system is absent, and that other prezygotic physiological barriers to selfing are either absent or incomplete.

Cytisophyllum sessilifolium and *Spartium junceum* after hand self-pollination trials produced no fruits and seeds, indicating the presence of a more effective prezygotic physiological control or self-recognition.

Sex allocation and pollen transfer efficiency. There is no trade-off between P/O ratio and the volume of single pollen grain, nor between pollen number and pollen grain volume (Charnov 1982). Our data are consistent with similar results obtained for Faboideae (Gallardo et al. 1994, López et al. 1999), but in contrast to other studies showing either an inverse (Vonhof and Harder 1995, Rodríguez-Riaño et al. 1999b) or a direct correlation (Ortega-Olivencia et al. 1997) between these parameters. Such contrasting evidence clearly shows that this aspect of reproductive biology of Legumes is still in need of deeper investigation.

A positive correlation has been found between pollen number, pollen biomass and ovule number, according to other studies concerning more restricted taxonomic groups (Small 1988, López et al. 1999). Our results suggest that a larger flower size implies more investment in the masculine function (P and V), but not in the feminine one (O).

We suppose that reproductive strategy is not affected by flower size as, in contrast to other studies (Small 1988), greater P/Os are not associated with larger flowers.

Explosive tripping is commonly considered the most advanced mechanism (Arroyo 1981) and lower P/O could be expected in certain species with explosive dehiscence (Dafni 1992). This hypothesis has been confirmed in Trifolieae (Small 1988), but not in Cytiseae (López et al. 1999). According to our data, tripping explosive mechanism is generally associated to relatively low P/Os, reflecting a higher specialization than pump (piston) or valvular types. The lowest P/O median value and variation range is associated to brush mechanism. It includes nectariferous species (Podda, personal observation), mostly of them belonging to tribe Vicieae, which comprehends also many herbaceous and possibly selfer species. Being nectar the principal reward offered to pollinators, we can hypothesize that in these species less pollen grains would get lost

during transfer to a compatible stigma, and thus a smaller number of pollen grains are required for ovule fertilization. Species with pump pollen release mechanism show the highest variability in P/Os. This category is represented by a very heterogeneous group of species which includes both nectariferous and polliniferous ones. Our data strengthen the hypothesis that pollen brush represents an advanced condition in the evolution of Faboideae, being linked with the major evolutionary trend towards a more efficient pollen transfer (Arroyo 1981, Leppik 1966 cited by Lavin and Delgado 1992).

Moreover, floral primary pollen presentation (tribe Hedysareae) is always associated to very high P/O values (Table 1), supporting Yeo's hypothesis concerning the higher efficiency of mechanisms with secondary pollen presentation (Yeo 1993, Howell et al. 1993, Cruden 2000). In spite of that, many other factors (morphological, ethological, environmental) influence the efficiency of pollen transfer (Dafni 1992, Cruden 2000, López et al. 1999, Jürgens et al. 2002, Parachnowitsch and Elle 2004), thus generalizations and simplifications should be avoided, especially within highly heterogenous taxa.

P/O ratios are different among tribes, with the lowest median values in Vicieae and the highest in Trifolieae; differences are significant between Cytiseae and Vicieae and between Cytiseae and Loteae. The P/Os are higher and variation range wider in Cytiseae, compared to Loteae and Vicieae. With few exceptions, Cytiseae are mostly polliniferous species, that offer pollen as main reward to pollinators (Polhill 1976; Bisby 1981; Vogel 1997; Rodríguez-Riaño et al. 1999a, 1999c; Galloni and Cristofolini 2003). By contrast, both Vicieae and Loteae comprise commonly nectariferous species, in which floral nectar is accumulated in two little openings at the base of the reproductive column (Vogel 1997, Rodríguez-Riaño et al. 1999c). The different pollination ecology can explain the higher P/O values characterizing polliniferous spe-

cies, as a consequence of the reduced efficiency due to removal of rewarding pollen by insects.

As previously mentioned the "pollen brush" is characteristic of tribe Vicieae, whose low P/O values can be justified also by the brush function in entomophilous pollination.

Annual life form is generally associated to "r-strategy", while perennials are commonly considered "k-strategists" (see, e.g. Primack 1979, Pugliese and Kozlowsy 1990). Annual species have a short period of time available to leave offspring, so that selfing brings an advantage to their fitness, assuring an efficient reproduction in a limited time. According to the "time limitation" hypothesis, predominantly selfing annuals are expected to have less reproductive allocation to pollinator attractants and smaller pollen:ovule ratios than facultative selfers or outcrossers (Aarensen 2000). Unlike annual species, perennials suffer major loss of fitness when offspring fail to outcross: they require relatively abundant pollen production to compensate for the loss resulting from inefficient vector transport or pollen consumption by the vectors themselves (Cruden 1977, Aarensen 2000). Our data are consistent with this hypothesis: pollen grain number and pollen:ovule ratio vary significantly between woody perennials and annuals, less significantly between perennial herbs and annuals. Among perennials, woody and herbaceous species do not differ significantly in pollen production or P/O. Similarly to previous studies on legumes and other plant families (Karron 1987, Small 1988, Rodríguez-Riaño et al. 1999b, Ben Brahim et al. 2001, Jürgens et al. 2002, but see López et al. 1999, Aarensen 2000), we found greater P/Os in perennials and significatively smaller ones in annuals, in agreement with the general assumption that annual species are often self-compatible, while perennials mostly outcrossing and self-incompatible (Arroyo 1981, Plitman and Levin 1990, but see Parachnowitsch and Elle 2004). Our results with *Colutea arborescens*, *Coronilla emerus* and

Hedysarum coronarium (perennials and self-compatible), are an exception to what is generally expected among legume species, demonstrating how variable reproductive strategies can be. Field tests indicate that insects visits are necessary for seed production in eight perennials, confirming the predictivity capacity of Cruden's P/O breeding categories in ecological sense (Cruden 1977). Xenogamous species have been defined by Cruden as primarily oucrossers, protandrous or self-incompatible, mostly requiring pollinators: this class of breeding system should include species with mechanical barriers to selfing (represented by the stigmatic cuticle), whose reproduction is strictly dependent on pollinator activity.

Appendix 1

Studied material. Sex allocation and pollen release mechanism were studied in all species. Productivity tests were conducted on a sample of species, during different years, as reported below.

Cytiseae Bercht. and J. Presl

Laburnum anagyroides Medicus.: Via Gaibola, San Lazzaro di Savena (Bologna), 23/04/2003 (BOLO 49050).

Cytisus hirsutus L.: San Lazzaro di Savena (Bologna), 23/04/2003 (BOLO 49040).

Cytisus scoparius (L.) Link: Monte Beni (Firenze), 15/06/2004 (BOLO 49059). Productivity: 2004 (sample size = 15 plants).

Cytisophyllum sessilifolium (L.) Lang.: Via Gaibola, San Lazzaro di Savena (Bologna), 17/04/2002 (BOLO 42010). Productivity: 2000, 2001, 2002 (sample size = 20, 19, 21 plants respectively).

Genista januensis Viv.: San Benedetto del Querceto (Bologna), 16/05/2003 (BOLO 49047).

Genista radiata (L.) Scop.: (1) Monte Beni, Covigliaio (Firenze), 14/06/2002 (BOLO 46440, BOLO 46442). Productivity: 2001 (sample size = 20 plants). (2) Monte La Nuda, Corno alle Scale (Bologna), (BOLO 46294, BOLO 46296). Productivity: 2000, 2001, 2002 (sample size = 15 plants each year).

Genista cilentina Valsecchi: (1) Ascea Marina (Salerno), 19/05/2002 (BOLO 46298). (2) Pisciotta (Salerno). Productivity: 2001, 2002 (sample size = 10 and 13 plants respectively).

Genista tinctoria L.: Monte Beni, Covigliaio (Firenze), 15/06/2004 (BOLO 49063).

Spartium junceum L.: Via Gaibola, San Lazzaro di Savena (Bologna), 12/06/2002 (BOLO 46292). Productivity: 2001, 2002 (sample size = 9 and 12 plants respectively).

Robinieae (Benth.) Hutch.

Robinia pseudoacacia L.: Via Angelo Custode (Bologna), 01/05/2003 (BOLO 49049).

Galegeae (Bronn) Torrey & Gray

Colutea arborescens L.: (1) Ca' de Mandorli, San Lazzaro di Savena (Bologna) 09/05/2003 (BOLO 49048, 49044, 49062); (2) Via Gaibola, San Lazzaro di Savena (Bologna), 10/05/2005 (BOLO 40467). Productivity: 2005 (sample size = 6 plants).

Astragalus glycyphyllos L.: Botanic Garden, Bologna, 07/06/2005 (BOLO 40465).

Hedysareae DC

Hedysarum coronarium L.: Via Dell'Eremo, San Lazzaro di Savena (Bologna), 01/06/2004 (BOLO 49053). Productivity: 2004, 2005 (sample size = 6 and 3 plants respectively).

Onobrychis viciifolia Scop.: Via Agucchi (Bologna), 04/05/2004 (BOLO 49058).

Vicieae (Adans.) DC

Lathyrus pannonicus (Jacq.) Garcke: Rocca Pitigliana (Bologna) 26/04/2003 (BOLO 49052).

Lathyrus latifolius L.: Ganzole, Pianoro (Bologna) 24/06/2003 (BOLO 49045).

Lathyrus aphaca L.: Via Dell'Eremo, San Lazzaro di Savena (Bologna), 01/06/2004 (BOLO 49057).

Lathyrus hirsutus L.: Via Dell'Eremo, San Lazzaro di Savena (Bologna),18/06/2004 (BOLO 49060).

Vicia hybrida L.: Ca' de Mandorli, San Lazzaro di Savena (Bologna), 01/05/2004 (BOLO 49043).

Vicia cracca L.: Ca' de Mandorli, San Lazzaro di Savena (Bologna), 24/05/2004 (BOLO 49055).

Vicia sativa L.: Via Angelo Custode (Bologna), 27/04/2004 (BOLO 49042).

Trifolieae (Bronn) Benth.

Trifolium alpinum L.: Malga Bocche, Paneveggio (Trento), 18/06/2003 (BOLO 48778).

Medicago arborea L.: Botanic Garden, Bologna, 05/04/2004 (BOLO 49041).

Ononis masquillierii Bertol. (= *Ononis spinosa* L. subsp. *masquillierii* (Bertol.) Greuter & Burdet): Via Dell'Eremo, San Lazzaro di Savena (Bologna), 18/06/2004 (BOLO 49061, 49066).

Ononis natrix L.: Via Gaibola, San Lazzaro di Savena (Bologna), 30/05/2005 (BOLO 40466).

Loteae DC

Lotus corniculatus L.: Via Angelo Custode (Bologna), 28/04/2003 (BOLO 49046).

Dorycnium hirsutum (L.) Ser.: Ca' de' Mandorli, San Lazzaro di Savena (Bologna), 24/05/2004 (BOLO 49054).

Securigera securidaca (L.) Deg. & Dorfl.: Ca' de' Mandorli, San Lazzaro di Savena (Bologna), 24/05/2004 (BOLO 49056).

Anthyllis vulneraria L.: Val Venegia, Paneveggio (Trento), 10/06/2004 (BOLO 49064).

Coronilleae (Adans.) Boiss.

Coronilla emerus L. (= *Hippocrepis emerus* (L.) Lassen): San Lazzaro di Savena, via Gaibola (Bologna), 19/04/2003 (BOLO 49051, 49039). Productivity 2004, 2005 (sample size = 11 and 10 plants respectively).

Coronilla varia L.: Via Dell'Eremo, San Lazzaro di Savena (Bologna), 28/06/2004 (BOLO 49065).

Hippocrepis comosa L.: Val Venegia, Paneveggio (Trento), 17/06/2003 (BOLO 48796).

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