

Full Length Research Paper

Analysis of floral Biology of *Vicia villosa* Roth. (Fabaceae)

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Received 17 December, 2011; Accepted 23 March, 2012

An analysis of the of sexual reproduction ability of *Vicia villosa* Roth. have been investigated. The biological material consisted of a tunisian cultivar (Sejenane) and two accessions originating from ICARDA (International Center of Agricultural Research in Dry Areas): acc. 2565 and acc. 3615. Pod formation rate (P/F), number of seeds per pod (SPP), and the number of empty pods per plant (NEP) were higher under free pollination than in self-fertilization without release, suggesting that this species is characterized by a preferential allogamy. That Allogamy seems to be ensured by a sporophytic auto-incompatibility. The gap between the number of seeds per pod (2.6 seeds on average) and the number of ovaries per gynaecium (4 ovaries on average) may reflect a fertilization malfunctioning in *Vicia villosa* Roth. The sporophytic auto-incompatibility, associated to both low pollinic germination rate (24 %) and flowers losses caused by gathering bumblebees (*Bombus terrestris* L.) constitute the main factors explaining the low fertility of this forage species.

Key-words: *Vicia villosa*, free pollination, self pollination, auto-incompatibility, pollinic fertility.

INTRODUCTION

According to available archaeological knowledge, vetch species would have been cultivated for the VIIIth century before Christ (Zohary and Hopf, 1988). The use of *Vicia villosa* Roth., began later during the IIIth century before Christ (Schulte-Motel, 1972). It is an annual Fabaceae with variable ploidy level ($2n = 2x = 14$ and $2n = 4x = 28$) (Coulot and Rabaute, 2009), belonging to tribe of *Viciae* (Kesavacharyulu et al., 1982; Maxted, 1995). As others pasture legumes, *Vicia villosa* is considered by agronomists as an improving physico-chemical soil quality species thanks to its atmospheric nitrogen symbiotic fixation ability and high crop residues return to the soil (Marouani, 1996; Hassen et al., 1998). Its role is also confirmed through the valorisation of dry regions in

numerous countries worldwide which are dominated by barley monoculture and provides a practical alternative to fallows, particularly, in low fertile lands. In Tunisia, hairy vetch is rare; its relative frequency is only about 7.7%. It is located on humid and sub-humid bioclimatic zones, on low altitudes (< 250 m), light textured and slightly acid to neutral soils (Hassen et al., 1998). *Vicia villosa* is little cultivated in Tunisia and even worldwide in spite of its agronomic advantages as legume partner in annual forage intercrop or mixture, its cold hardiness and its ability to valorise different substrata in the regions where common vetch (*Vicia sativa* L.) does not perform well and does not fit the new farm requirements. The major obstacle that hampers hairy vetch utilization in crop rotations resides in its weak seed yield potential. The average seed yield is actually about 500 kg ha⁻¹ which represents the fourth of the seed yield potential of common vetch. Low seed yield seems to be related to the low pod formation rate (only one flower out of 15 can

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Table 1. Main characteristics of studied plant material

Populations	Thousand Seed Weight (g)	Earliness	Corolla colour	Origin
Var. Sejenane	24.8	Late	Violet	Tunisia
Acc. 2565	36.9	Early	Purple	ICARDA
Acc.3615	52.6	Early	Purple	ICARDA



Figure 1. Metaphase of *V. villosa*, observed on root mitoses, coloured with Feulgen. (Photo taken by a 10 megapixels digital camera (Zoghalmi et al., 2009))

produce a pod) and, to the considerable number of flowers abortion caused by a defective release of wild bees such as bumblebees, *Bombus terrestris* L. (Hassen, 1999). The objective of this research was to quantify the reproductive potential of three populations of *Vicia villosa* from different origin and precocities under self and free pollination. The fertility of plants was measured by the frequency of pod formation, the average number of seeds per pod, and the number of empty pods by plant. These parameters were used by other authors on several botanical species such as *Medicago sativa* L. (Dattée, 1975), *Hedysarum coronarium* L. (Baatout, 1982; Chriki, 1986), *Lathyrus sativus* L. (Chaibi et al., 1985; Ben Brahim, 2000; Ben Brahim et al., 2001), and other pasture legumes (Zoghalmi Khéilil, 2005).

MATERIALS AND METHODS

Plant material

The plant material used in this study consisted of a local variety named Sejenane (Vv1) and two accessions introduced from ICARDA: acc. 2565 (Vv2) and acc. 3615 (Vv3). The variety Sejenane is a local variety which was selected via agronomic evaluation from a local germoplasm originated from Ras Rajel region (humid climate with annual rainfall ranging between 800 and 1000 mm and minimal average temperature is of 6.3°C). The two accessions 2565 and 3615 were provided by ICARDA in the frame

of germoplasm exchange. All this material was conserved at the National Institute of Agronomic Research of Tunisia (INRAT). The main characteristics of the plant material are given in table 1. Karyological analysis showed that the three populations are diploids with a karyotype of $2n= 2x= 14$ chromosomes. Chromosomes of *V. villosa* have homogeneous sizes, varying from 5.8 and 6 μm for the shortest and the longest chromosome, respectively (Zoghalmi et al., 2009) (figure 1).

Seed germination

Seeds of all populations were placed for germination in *Petri* dishes on filter paper soaked with distilled water under ambient temperature. Seeds were initially hand scarified (to enhance germination) and then washed with hypochlorite (to prevent them from further fungal contamination). After 4 or 7 days (according to population), the seedlings were transplanted into twenty litres capacity plastic pots (with a seeding density of 4 seedlings per pot). The pots contain a substratum constituted by 2/3 of sand and 1/3 of peat, sterilized under a temperature of 105°C during 48 hours in order to destroy fungi and other potential soil pathogens. The experiment was arranged in a completely randomized block design with 3 replications. Experimental unit is constituted by 5 pots containing 4 plants each, giving a total number of 20 plants by variety. Weeds removal, irrigation, and harvesting operations were hand made when necessary.

Plant fertility parameters

In order to estimate the fertility of *V. villosa* populations, three parameters were measured:

- Rate of flowers producing pods (noted P/F ratio): the chosen inflorescences for the determination of this parameter are marked at full blooming stage and were harvested separately at maturity.
- Number of seeds per pod (noted SPP). This parameter is determined on a sample of 50 pods taken randomly on each plant at physiological maturity stage.
- Number of empty pods per plant (noted NEP) by counting the number of empty pods at harvesting.

The mean for the population is obtained by dividing total number of empty pods by the number of plants constituting the studied genotype.

Studying of the pollination mode

We have imposed experiments of self and free pollination on several plants per population to appreciate reproduction ability of the studied genotypes. As flowers of *V. villosa* are grouped in inflorescences, the plant can carry at the same time during flowering period, buds, opened, unopened flowers, and young green pods. In these two experiments, only the unopened flowers were manipulated. For each experiment, ten flowers per plant and 20 plants per population were manipulated.

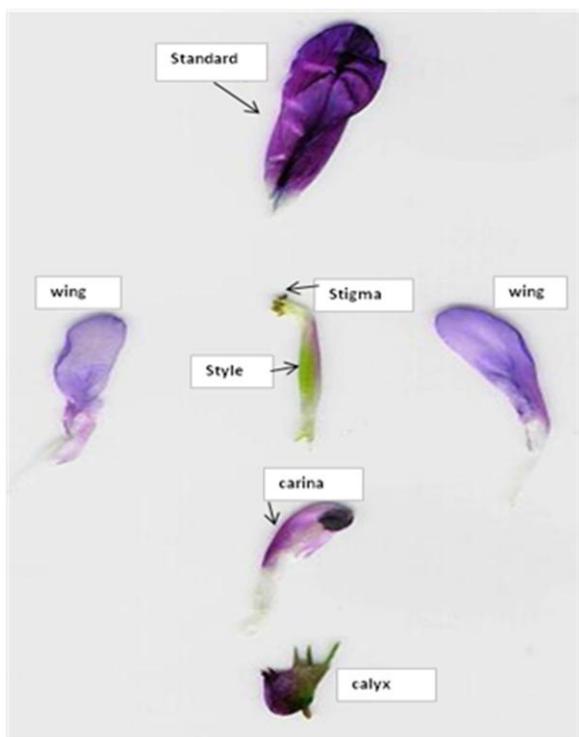


Figure 2. Perianth of *V. villosa*

Experiment of self pollination without release

The floral buds are bagged with perforated cellophane to avoid any intervention of pollinating agents such as bees. The technique of plant isolation or bagging of flowers was often used as classic method for self-pollination of vegetables presenting a competition between self and foreign pollen. This technique was used previously by Demarly (1963) on *Medicago sativa*; Hassen (2000) on *Vicia narbonensis* L., and Zoghalmi khéilil (2007) on *Astragalus hamosus* L. and *Coronilla scorpioides* Koch. L. An average of 10 flowers per plant and 20 plants per genotypes were used to carry out this experiment.

Experiment of free pollination

In this experiment, flowers were chosen and marked at budding stage and then left for free pollination up to maturity. As for most of forage and pastoral legumes, the inflorescences of the hairy vetch are visited by pollinating agents (Morthorpe and Jones, 1988). These pollinators are represented by the domestic bee (*Apis mellifera* Linnaeus, 1758 (Apidae).) and a species of bumblebee (*Bombus terrestris* Linnaeus, 1758 (Apidae)) which was proven to be specific to several forage legumes such as *Vicia* spp. *Medicago* spp. (Hassen et Seklani, 1990). -

Statistical analysis

The analysis of variance (procedure GLM) was used in order to determine the effect of the genotype and the mode of pollination on the variation of measured parameters. For comparison of means, the test of Duncan was performed using the program SAS (SAS, 1990).

RESULTS AND DISCUSSION

Flower morphology and development

Before approaching the experimental study on reproduction mode of the three genotypes of *V. villosa*, it is important to underline some preliminary observations relative to the development and the morphology of the flower. Flowers of *V. villosa* are 14 to 18 mm long and are gathered in racemes (in a number of 12 to 30), elongated and are as longer as the corresponding leaf. The corolla from 12 to 18 mm is purplish for the two accessions Vv2 and Vv3 while for the variety Sejenane (Vv1) the corolla presents white spots on its extremity. Both wings shelter the hull opened on its extremity. The standard covers partially the wings and the keel and fall at anthesis. Its limb is shorter than its claw. In this case, stamens and stigma are not protected from the intervention of pollinators.

During the blooming stage, the standard edges get over and the flower remains in that state during 3 to 5 days allowing the pollinators to intervene. The ovary, constituted by a variable number of ovules depending to the population, is surmounted by a spindly style, intensely hairy in the 2/3 of its length, ending in the bottom of the keel by which it follows the curvature. The hairy stigma on its frontal faces forms a species of a spoon in which settles the pollen. The androecium is formed by 9 stamens united by their fillets on the 2/3 of their length and 1 free stamen. These stamens are wrapped in a stamina membrane opened by its extremity superior what leaves anthers near to the stigma. The observation of floral buttons of diverse ages showed that the dehiscence of anthers and the deposit of the pollen on the stigma are premature and occur before blooming. The necessity of understanding and following the flower development of *V. villosa*, imposed an examination of several stages of the floral morphogenesis, from the new floral bud to the faded flower (figure 2).

The stages of flower development

The examination of the buds since their appearance until blooming, allowed us to identify 5 different stages during floral development of *V. villosa* populations (figure 3):

- Stage 1: closed buds
- Stage 2: hooded buds
- Stage 3: half-opened flower
- Stage 4: spread flower
- Stage 5: bloomed flower

The analysis of the floral biology was based on the interpretation of stages 2, 3 and 4. Stages 1 and 5 (closed bud and faded flower) were omitted in this analysis because they do not represent interesting information on the aptitude of sexual reproduction of *V. villosa* populations. The description of the various floral stages was noticed on bagged and isolated flowers.

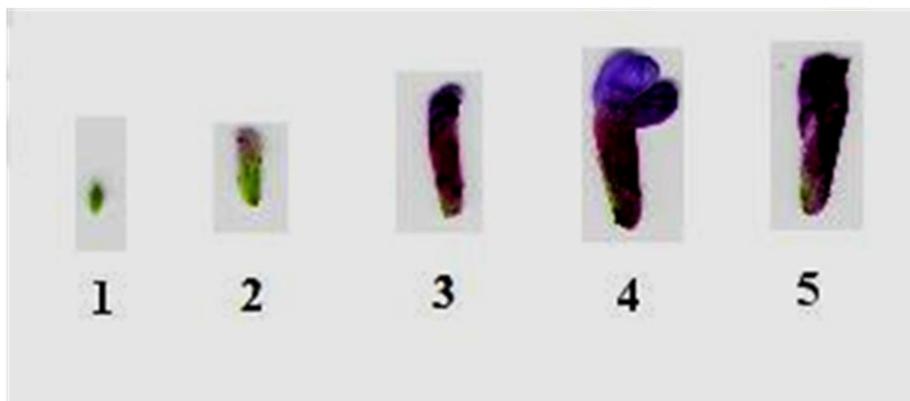


Figure 3. Different stages of development of the flower in *V. villosa* (1= closed bud, 2= hooded bud, 3= half-opened flower; 4= spread flower, 5= bloomed flower)

Stage 2

The bud size is variable according to the population. It reached 5, 5.2, and 6.1 mm long for Vv3, Vv2, and Vv1, respectively. The corolla, hardly visible, is of whitish colour. The calyx, which wrapped two thirds of the bud is green, gamosepalous and pubescent with unequal teethes of 3 mm long. The dissection of the flower bud showed an ovary with one carpel containing green ovules more or less turgescient having variable forms and sizes. The pistil is straight and slightly curved on the top. The stigma is not sticky, slightly pubescent and free of pollen on its surface. The androecia present stamens with anthers slightly dehiscent of whitish colour charged of immature pollen grains having greenish colour.

Stage 3

The flower is half-opened, its length averaged 13.5, 13.8, and 15.8 mm for Vv3, Vv2, and Vv1, respectively. The corolla, partially emergent, contains two wings, a carina with pale pink colour and a standard with variable colour: purple for Vv3 and Vv2 and violet for Vv1. The keel is semi-opened. The calyx, which wraps the corolla for third of its length, is gamosepalous, with unequal teeth spotted with anthocyanin on its circumference. The ovary is straight and supplied with one carpel, the style is more rectilinear than in the previous case, with extremity is slightly curved. The stigma, hairy and sticky, presents grains of not germinated pollen. Anthers, yellow, filled with grains of mature pollen, are dehiscent.

Stage 4

It corresponds to the opened flower of about 15 mm long on average. The corolla separated completely from

sepals, is bi-coloured: purple standard (Vv3 and Vv2) and violet (Vv1) with pink wings. The keel is opened and exposes the flower for pollinators. The calyx is well developed; it shows unequal teethes and anthocyanic spots on its circumference. The stigma is sticky and translucent, strongly bristled by long, dense and muddled hairs, on which settle grains of not germinated pollen. Stamens, wrapping completely the stigma, carry anthers of grey or black colour, dehiscent, shrivelled and without pollen.

From previous paragraphs, we can deduce that for *V. villosa*, the observation of floral buds of diverse ages (stages 2, 3 and 4) showed that the dehiscence of anthers and the depositing of the pollen on the stigma are premature and occurred before flower blooming. However no pollen germination occurred on the stigma. It seems that the auto pollen is unable to germinate on its own stigma. This phenomenon, known as sporophytic auto-incompatibility, is very frequent in forage legumes as alfalfa (Hassen, 1990), and white clover (Wyatt, 1983).

Analysis of reproduction mode of *V. villosa*

To understand the sexual reproduction aptitude of the three populations of *V. villosa*, we proceeded to analyse certain aspects of floral biology. This study is fundamental step before initiating a breeding programs and elaborating appropriate selection scheme.

In self and free pollination, plants fertility was appreciated through pods formation frequency (noted P/F ratio), number of empty pods per plant (NEP), and number of seeds per pod (SPP). These three parameters were used on alfalfa (Dattée, 1975), tomato (Rick et al., 1978), sainfoin (Baatout, 1982) and grass pea (Chaiebi et al., 1985; Ben Brahim, 1990) in order to study the capacity of the sexual reproduction in this species. In both self and free pollination, we manipulated, 4975

Table 2. Analysis of variance of the proportions of flowers giving pods (P/F), number of empty pods (NEP), and the number of seeds per pod (SPP), measured on Vv1, Vv2, and Vv3 populations of *V. villosa*, under free and self pollination without release.

Measured Parameters	Average	observed F		Signification	
		Mp	Pop	Mp	Pop
P/F	0.32	867.27	32.97	HS	HS
NEP	0.75	0.48	13.75	NS	NS
SPP	2.67	7.98	6.27	HS	HS

*Mp= factor 'Mode of pollination'; Pop= factor 'Population'

Table 3. Duncan's classification for the parameters P/F, NEP and SPP in three populations of *V. villosa* under self pollination without release

Populations/parameters	Vv1	Vv2	Vv3
P/F	0.03c	0.05b	0.07a
NEP	1.91a	2.64a	2.209a
SPP	7.73a	2.61b	1.89b

P/F: proportions of flowers giving pods, NEP: number of empty pods, SPP: number of seeds per pod.

flowers randomly taken from Vv1, Vv2, and Vv3 populations of *V. villosa*. These flowers have produced 1592 pods. The proportion of flowers giving pods, whatever the mode of pollination averaged 32 %.

Analysis of variance applied to these parameters, showed a highly significant effect of the population on P/F, NEP and SPP. Mode of pollination effect was significant only on P/F and SPP (table 2).

Compared with other species such as *Vicia narbonensis* (Hassen, 2003), *Lathyrus cicera*, and *Lathyrus ochrus* (Ben Brahim et al., 2001), the P/F obtained in this experiment (whatever the mode of pollination) is relatively low (0.32 vs. 0.96 in *Lathyrus cicera*). The SPP, with an average of 2.67 seeds per pod, is very low compared to that observed in *Vicia narbonensis*, 4.71 seeds/pod (Hassen, 2003) and *Lathyrus ochrus*, 3.51 seeds/pod (Ben Brahim et al., 1990). These results showed the weak performance of this species as regard to fertility.

Self pollination without release

The results of this experiment are presented in table 3. Self pollination produced variable fertile flowers number (P/F) between the three populations. For Vv1, the rate of autogamy is very low (0.03), significantly lower than that produced by the introduced accessions Vv2 and Vv3s. The best rate of fertility in self-pollination is observed for Vv3 population (0.07). The average number of seeds per pod varies from 1.91 to 2.64. The lowest value characterizes the population Vv1 (1.91 seeds) while the

populations Vv2 and Vv3 presented respectively, 2.64 and 2.29 seeds per pod. This result is in agreement with that obtained by Ben Brahim et al. (1990) with *Lathyrus sativus* for which they estimated SPP to be between 1.58 and 2.07. For the NEP, the population Vv1 is the low fertile with an average of 7.73 empty pods by plant. It surpasses significantly, the populations Vv2 (NEP = 2.61) and Vv3 (NEP= 1.89). Taking into account these averages, it seems that *V. villosa* has few empty pods in comparison to other fabaceas. With an average of 4.03 empty pods by plant, it shows itself more successful, for this character, than *Vicia narbonensis* in which the NEP can reach 7 empty pods per plant (Hassen, 2003) and *Vicia sativa* in which the NEP can reach 11 empty pods per plant under self pollination without release (Kahlaoui et al., 2009).

The free pollination

For *V. villosa*, as for other pasture legumes such as, *Lathyrus* spp., *Medicago* spp., *Astragalus* spp., *Trifolium* spp., natural pollination is entomophilous. It is mainly ensured by bees and bumblebees (Valero et al., 1985). In a trial studying the efficiency of insect pollination of alfalfa in Tunisia, Hassen and Seklani (1990) showed that the installation of an apiary constituted by 10 colonies of bees per hectare, at flowering stage, had increased alfalfa seed yield from 180 kg ha⁻¹ (close to national average yield) to 600 kg ha⁻¹. The fertility parameters under free pollination of three populations of *V. villosa* are recorded in the table 4.

Table 4. Duncan's ($\alpha=5\%$) classification of three populations of *V villosa* Roth for the parameters P/F, NEP and SPP under free pollination.

Populations/parameters	Vv1	Vv2	Vv3
P/F	0.35a	0.34a	0.28b
NEP	0.42b	0.31b	1.42a
SPP	3.56a	3.34a	2.17b

P/F: proportions of flowers giving pods, NEP: number of empty pods, SPP: number of seeds per pod.

Several previously marked inflorescences from plants belonging to all studied populations (Vv1, Vv2, and Vv3) were submitted to the action of the pollinating agents. Under this treatment, results showed superior proportion of fertile flowers. It reached 32 % on average *versus* 5 % recorded in the experiment of self pollination without release (table 3). Nevertheless, one should stress out that under natural pollination, stigmas, besides the pollen coming from the same flower (auto pollen), can also receive pollen from flowers belonging to other plants (cross pollen). The latest one seems in our case, more active than the auto pollen.

The proportion of flowers leading to pods (P/F) varied significantly between populations. P/F ratio averaged 35%, 34%, and 28% for Vv1 and Vv2 and Vv3 populations, respectively (table 4). For the three populations, P/F ratios obtained under free pollination are significantly higher than those recorded under self-pollination. This increase can be explained by a competition in favour of cross pollen.

Within each population, number of empty pods (NEP) varies considerably between individuals from 0 to 15 empty pods per plant. The Vv3 population presented the highest NEP of 1.42 surpassing significantly the populations Vv1 and Vv2 with NEP of 0.42 and 0.31 respectively. The results obtained for P/F and NEP, showed the advantageous of the local variety (Vv1) concerning the capacity to the sexual reproduction; the rate of pod formation and the NEP were in favour of this variety in comparison with to the introduced accessions.

The average number of seeds per pod in natural pollination is 3.02. It varied, from 2.17 (Vv3) to 3.56 (Vv1). The accession Vv2, with 3.34 seeds per pod is the most performing one (table 4). The fertility measured through the number of seeds per pod was higher in free pollination than in self-fertilization. The mechanisms favouring the formation of seeds could be related to selective advantage of the cross pollen. Bonciarelli (1960) and Demarly (1963) showed that for *Medicago sativa*, self-fertilization occurred with a maximum frequency of 6 % where the cross pollen ensured the quasi-totality of the fertilization.

We can conclude that under natural pollination, frequency of pod formation and number of seeds per pod

are high in comparison with those obtained in self-fertilization without release. For three populations, the parameters P/F and SPP, expressed in the case of *V villosa*, a regime of reproduction preferentially allogamous. The proportions of self pollinated flowers, estimated to 5 % were mentioned in other allogamous species such as *Medicago sativa* (Demarly, 1963) and *Lathyrus latifolius* (Valero et al., 1985). However, it is necessary to emphasize that the frequencies of pod formation, the number of seeds per pods, and the number of empty pods were variable between populations. Those populations seem to be polymorphic as regard to the fertility. This resulted from the genomes diversity between the different accessions.

Average number of ovaries by gynaecium was equal to four while number of seeds per pod from the same inflorescences averaged 2.6. It appeared that germinated pollen is unable to fertilize all the present ovaries in the pistil. The gap between both parameters (number of ovules and number of seeds per pod) may reflect a fertilization dysfunction in *V. villosa*. This result was revealed by Siemens and Johnson (1995) in *Medicago sativa*. The authors showed that the imperfect fertilization was the main factor limiting the number of seeds per pod. Pechan (1988) revealed that pollen germination on the stigma did not guarantee the formation of seeds even when pollen tubes were present in the region of the micropyle.

CONCLUSION

The results obtained for all the examined parameters showed that *V. villosa* ., knows important difficulties to reproduction. Investigations at the genetic levels (study of the meiosis) as well as molecular level can clear up the real problems of the decline of fertility in this species. The values obtained for the rate of pod formation (5- 32 %) and the number of seeds per pod (2.2 – 3.02) in case of *V. villosa* are relatively low in comparison with those recorded in some fodder legumes such as perennial lucerne, grass peas, and vetches where the proportions of flowers giving pods can go up to 80 % and the average SPP can reach 6 seeds per pod. These values bring out

the dysfunction of all the processes governing the pollination and the fertilization of hairy vetch. The sporophytic auto-incompatibility associated, on one hand, to the low pollinic germination (24 %) and to the losses in flowers caused by the leasing of bumblebees (Hassen, 2009), on the other hand, are factors which can explain, at least partially, the low fertility of *V. villosa* ..

However, the improvement of seed production in *V. villosa* is possible because the experiments realized on allogamous species in which sexual reproduction presents specific difficulties (such as the system of release in *Medicago sativa* for example) have supplied methodologies as well as interesting results on yield components such as the P/F, the SPP, the NEP and seed yield itself (Hassen and Seklani, 1990). Previous works, undertaken on technical crop essentially, were based on the intensification of the role of the pollinating solitary bees of these allogamous legumes. The transposition of this methodology in *V. villosa* will pass, in our opinion, by the respect of some practical rules such as:

i. avoid the competition of the adventitious and the 'melliferous trees and shrubs (*Eucalyptus*, *Acacia*, *Rosmarinus*) who exercise a stronger attraction than do the hairy vetch (relatively little supplied with nectar). This implies that a crop of *V. villosa*, exempt of weeds and placed so far from melliferous plants, can bring a substantial improvement of seed production of this species whose seed yield reaches at present 8 qx.ha⁻¹.

ii. the establishment of an apiary of domestic bees is also proposed. Hassen and Seklani (1990) revealed that the contribution of an apiary placed in a lucerne crop has improved significantly the presence of bees whom density increase from 4 to 48 individuals by m² and also the rate of release (pollination rate) of the crop which passed from 8 to 17 %. According to Dirk (1995), the wild and social pollinating fauna has really participate in the pod formation in species of the genus *Vicia* and particularly in *V. villosa*, where the author revealed a percentage of allogamy by more than 90%.

Few works are recently devoted to the treated subject. In this study, we attempted to judge consequences of some phenomena that may explain *Vicia villosa* low fertility without giving evident explanations. So, more investigations at genetic and molecular level are needed to go more thoroughly into the comprehension of mechanisms affecting the fertility of *V. villosa* specimens.

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