Methods to study the advantages of cleistogamy in oilseed rape in limiting unwanted gene flow

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Abstract

The interest of cleistogamy (permanent closing of flowers) lies in two ways: to favour self pollination (i.e. to maintain pure lines) and to reduce gene flow by limiting pollen dispersal. The latter aim could be very beneficial in the case of the GMO control.

Presently, the stability of the cleistogamous trait (obtained by mutagenesis) is attained in several lines but has to be confirmed under various climatic and agricultural conditions.

In addition, stable cleistogamous lines having an erucic acid marker are now available. This marker can be easily detected in the seeds and its content found in the offspring seeds is a good indicator of cross-pollination. Thus, biological material now exists enabling several experimental designs to study the impact of this trait both on autogamy rate and pollen dispersal.

Two kinds of experiments can be carried out in the field:

- To study the incidence of cleistogamy on the autogamy rate in the cleistogamous plants, a small proportion of seeds of one cleistogamous line is sown in a field of a conventional oilseed rape having a high erucic acid seeds content as a marker.
- To study the role of the cleistogamy on the pollen transfer to non cleistogamous plants, on the opposite, a small proportion of seeds of a conventional oilseed rape exhibiting a low erucic acid level is sown in a field of a high erucic acid cleistogamous line.

A third kind of experiment can be designed to study the effect of cleistogamy in escaping allo-pollen contamination according to the distance of a contaminant neighbouring plot.

In addition, because honeybees are involved in pollen transfers their foraging behaviour on closed flower is investigated in order to known whether they open the flowers or not in the field experimental conditions.

Key words: Oilseed rape, *Brassica napus*, cleistogamy, honeybee, gene flow, co-existence

Introduction

Conventional oilseed rape is a hermaphrodite plant with a highly variable autogamy rate. The autogamy rate seems to mainly depend on the genotype but also on the environmental conditions. On the other hand, intraspecific but also some interspecific cross-pollination can occur by pollen dispersal due, in various proportions, to wind or pollinating insects (mainly honeybees) according to the situation. Thus the interest of cleistogamy (permanent closing of flower) lies in two ways:

- To favour self pollination
- To reduce gene flow by limiting pollen dispersal

The cleistogamous trait as been selected from induced mutagenesis in oilseed rape and patented (Renard & Tanguy, 1997). The trait is controlled, in major part by one gene (Clg1) and a positional cloning project is in progress to isolate this gene (Genoplante Project).

Since 1988, several field experiments have been carried on cleistogamous lines in order to study their impact on pollination. In particular the evaluation of the benefits brought by cleistogamy to increase harvest purity in several cropping systems has been simulated using a spatio-temporal model: the GENESYS rape (Colbach et al, 2001; Colbach et al, 2004). Although some results were impaired by the lack of stability in the newly created lines, a positive effect of cleistogamy on the autogamy rate was strongly suggested (Fargue et al, 2006). Presently, lines with a stable cleistogamy exist but a verification of the stability of the trait under several climatic and agricultural conditions are needed.

The second aim is to verify the impact of cleistogamy both on autogamy and pollen dispersal limitation under several hazard conditions i.e. respectively when cleistogamous flowers are placed in a high airborne pollen concentration emitted by an unwanted variety or when some volunteers of normal oilseed rape are flowering in a field sown with a cleistogamous line having particular genetic specificity (for instance brought by a transgene).

Another aim is also to measure the autogamy rate of a cleistogamous line according to its distance from a neighbouring and contaminating pollen source composed of a classical cultivar having a high erucic acid marker.

A third part of our study deals with the pollinating insects. Previous studies have been undertaken on the impact of the closing of the flowers on the pollinating insects' behaviour. It has been shown that honeybees and bumblebees visit closed flowers only to probe nectar to make honey (Pierre & Renard, 1999) but do not open them to collect pollen. Acting so, they have no contact with the reproductive organs of the closed flower and have not a high impact on the pollen dispersal (Pierre et al, 2002). This typical behaviour is observed when the insects are foraging in a homogeneously cleistogamous field.

Nevertheless, their behaviour is still unknown when some cleistogamous plants are placed in a conventional field and reciprocally, i. e. when classical flowers are scarce and look unlike the major celistogamous plants present in the field. This has to be taken into account to reliably evaluate the efficiency of cleistogamy on plant containment and coexistence.

Material and Methods

Measure of the stability of the cleistogamous trait under several conditions

The aim of this protocol is to check stability of cleistogamous trait during flowering among 2 genetic backgrounds (lines Clg A and Clg B) compared to 2 commercial leading cultivars. Experiments are carried at 5 locations in UK, Germany and France (European project "Co Extra"). In order to estimate the local climatic and pedoclimatic effects, the experimental sites are placed near weather station able to provide rain falls, max and mini daily temperatures, and relative humidity. In addition, situation (valley, plain, slop, plateau...), type of soil (texture, amount of stones or soil analysis if available), estimation of the water availability in the field are considered. Trials are managed following classical farmers technical practices and several parameters are recorded from sowing to harvest: sowing date, seeds per m2, elementary plots surface, fertilization, treatments during vegetation especially fongicides and growth regulators (chemical, date, quantity), harvest date.

Observations are made at different vegetative phases of the plant: regularity of plant density in Autumn or Winter, fresh aerial weight / m², phoma leaf spots, date of stage C2 and F_1,height at stage C2, G2 index, pest and disease, lodge, seed weight at harvest.

The key observation is the "opening level" of flowers on primary and secondary stems at flowering. These observations are made weekly on plots.

The opening level is noted from 1 to 5**.** Notation concerns the whole mature inflorescence. Note 1 corresponds to a completely opened inflorescence similar to a conventional open oilseed rape; note 3 corresponds to half opened and note 5 corresponds to a completely closed inflorescence. A flower is noted as closed when petals are grouped making a cone and looks as a big yellow bud. A particular pattern exists: some flowers have grouped closed petals but there is one lateral gap between them. When several flowers of the inflorescence are opened in such a way the notation can be considered 3* (* indicating this particular form of opening). Because the opening level can vary greatly according to several factors, notations will be done at the beginning of flowering, at full flowering and at the end of flowering under various conditions. For the same reasons, notations will be performed in the morning and in the afternoon of the day. In case of variability among plants in the plot, 20 plants per plot are considered.

Measure of the impact of cleistogamy on autogamy and pollen dispersal

Experimental design 1: study of the autogamy rate

The experimental design consists in a 1ha field sown in major part (96%) with a conventional oilseed rape cultivar 'cv. H' having a marker: a high erucic acid seed content. A minor part (2%) is sown with a low erucic cleistogamous line (Clg A) and another part (2%) with a low erucic male-sterile line of the cultivar 'ms cv. L'. In addition several test plants of the male-fertile cultivar 'cv. L' are haphazardly sown in the field. All these plants ('ClgA', 'cv. H', 'cv. L', 'ms cv. L') are freely pollinated by insects or wind and 40 of them, randomly chosen, are marked and harvested at the end of the experiment. In order to make comparisons between open-pollination and controlled pollinations (self-pollination and cross-pollination) other plants of the 4 genotypes are maintained under individual pollen proof cage for self-pollination (30 plants per genotype) whereas cross-pollinations are manually made between 'cv. H' used as male and respectively 'cv. L', 'ms cv. L' and the emasculated line Clg A used as females.

Experimental design 2: study of the pollen dispersal

In order to investigate the ability of cleistogamous flower to reduce pollen dispersal, cleistogamous lines with a high erucic acid seed content as marker have been created by INRA.

The experimental design consists in a 1ha field sown in major part (96%) with a cleistogamous oilseed rape line 'Clg H' having the marker. A minor part (2%) is sown with a low erucic conventional cultivar 'cv. L' and another one 2% with a low erucic male-sterile line of the cultivar 'ms cv. L'. All these plants ('Clg H', 'cv.L', 'ms cv. L') are freely pollinated by insects or wind and 40 of them, randomly chosen, are marked and harvested at the end of the experiment. In order to make comparisons between open-pollination and controlled pollinations (self-pollination and cross-pollination) other plants of the 3 genotypes are maintained under individual pollen proof cage for self-pollination and control (30 plants per genotype) whereas manual cross-pollination are made between 'Clg H' used as male and respectively 'cv. L', 'ms cv. L' used as females.

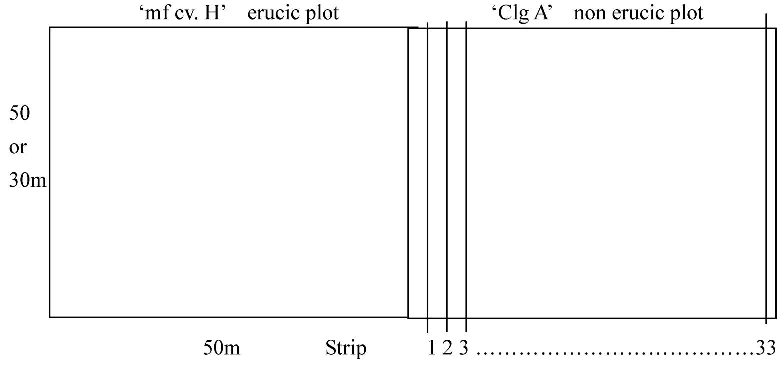
Measure of the autogamy rate of a cleistogamous line according to the distance from a contaminating pollen source

The objective of the protocol (European Project "Co Extra") is to measure the autogamy rate of a cleistogamous line neighbouring a classical plot of a cultivar having a high erucic acid marker.

The trial (in an isolated field, and replicated at 3 sites: France, UK and Germany) consists in 2 big neighbouring plots (50m length×50 or 30m width, each):

- one sown with the cleistogamous line ClgA
- one sown with a mixture of 99% 'mf cv. H' (high erucic cultivar = erucic plot) used as contaminating pollen source and 1% of Clg A in order to estimate the rate of autogamy of isolated cleistogamic plants inside the erucic canopy.

Each plot is constituted of 33 belts of 5 sowing rows (1.5m).



The autogamy rate is estimated through erucic acid content of seeds of the cleistogamous line issued from self or allo pollination. The measure is done according to the distance from the erucic plot at 10 belts: n°1, 2, 3, 4, 5, 15, 30, 31, 32, 33 i.e. respectively at 0, 3, 4.5, 6, 7.5, 22, 45, 46.5, 48 and 50m distance.

Controls are made on: 30 self-pollinated 'ClgA' plants (bagged), 30 self-pollinated 'mf cv. H' (bagged),

30 manual cross-pollinated plants (female ClgA×male 'mf cv. H'), 30 open-pollinated ClgA plants representing 1% of plants inside the erucic plot and 80 (8 plants×10 belts) open-pollinated Clg A plants in the Clg A plot.

Method: in the three experiments, the erucic acid seed content is measured by Gas Chromatography in seeds produced respectively by open- pollination, self-pollination or manual cross-pollinations. In every sample, the analysis is made on a mix of 20 seeds (or at least more than 500mg of mixture).

Study of the foraging behaviour of honeybees

A 10 combs Dadant hive is placed in the field at the beginning of flowering. The number of foraging honeybees is regularly recorded on 3 belt transects (10m length) and meanwhile the number of available flowers is measured at the same places. From these data the number of honeybees/1000 flowers is evaluated to verify if their foraging activity is enough to ensure insect pollination. Individual honeybee foraging behaviour is studied in order to know if foragers visit scarce cleistogamous flowers when they are surrounded by normal flowers (Experiment1) or, on the opposite, when scarce normal flowers are surrounded by cleistogamous flowers (Experiment2). The observations are made in a circle of 0.56m diameter (# 0.25m²) with in its center a cleistogamous (Experiment 1) or a normal flower (Experiment 2). Observations are made from place to place in the field. The number of normal and cleistogamous available flowers in the observation area is evaluated and compared to the number of visit to normal and cleistogamous flowers by the honeybee during one minute. The numbers of passage between both types of flowers are also recorded. Meanwhile, the individual foraging behaviour is registered: i) crawling over posture for pollen and/or nectar foraging (inducing high pollen loading and pollen deposition), ii) inserting lateral posture for nectar foraging (no contact with anther = low pollen transfer), iii) attempt to open the closed flower to forage for pollen.

Conclusions

The first step consisting in a verification of the stability of the cleistogamous trait will be extensively studied in very various conditions. This phase of our study is necessary if commercial production of cleistogamous varieties is looked in the future. The second step is to verify the benefits of cleistogamy on autogamy and /or reduction of pollen dispersal. The 3 experimental designs used correspond to the highest risk of contamination (high airborne allo-pollen concentration). In addition, the risk of pollen dispersal due to the foraging behaviour of insects is taken into account. Concerning the method used, the main point is to reliably evaluate the average erucic acid content (± standard error) of the pure seeds, the seeds issued from manual cross-pollination in order to proportionally determine what the autogamy rate (1-allogamy rate) due to free pollination is in every case.

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