CO-EXTRA

GM and non-GM supply chains: their CO-EXistence and TRAceability

Project number: 007158

Integrated project
Sixth Framework Programme
Priority 5
Food Quality and Safety

Deliverable D1.2

Title: Decision on the relevance of potential biological mitigation techniques / Selection of those techniques that are developed and/or validated further

Due date of deliverable: M 12

Actual submission date: M 13

Start date of the project: April 1st, 2005 **Duration**: 48 months

Organisation name of lead contractor: BBA

Revision: V1.0

Project co-funded by the European Commission within the Sixth Framework Programme (2002-2006)	
Dissemination Level	
PU Public	
PP Restricted to other programme participants (including the Commission Services)	**PP**
RE Restricted to a group specified by the consortium (including the Commission Services)	
CO Confidential, only for members of the consortium (including the Commission Services)	

1 Summary

WP1 is aimed at assessing and developing biological tools and methods to allow producers to grow kind of crops they choose with minimised risks of admixture between GM, conventional and organic products. Therefore, the objectives of WP1 during the first year was to analyse and validate methods for restricting gene flow during cultivation by field experiments located at different sites in Europe with CMS-maize and cleistgamic rapeseed in order to investigate the stability of these biological mitigation techniques. In addition, field trials has been carried out in order to investigate the abilities and potential use in a Plus-Hybrid system of a large set of modern CMS-maize hybrids in Switzerland and France. As a result, ring experiments (2006) with validated CMS maize and cleistogamic rapeseed lines has been planned for Switzerland, Germany, Bulgaria, France and UK.

2 Biological mitigation techniques

2.1 Cytoplasmic male sterile maize

The experiments performed during the first year had the objective to characterise maize cms hybrids from the types cms-C, cms-S and cms-T with respect to the degree and stability of the sterility, in dependence of environmental factors. For this, twenty modern hybrids from different maturity groups and different cms types were requested from different European breeding companies. All twenty hybrids were sown at three to four different sowing dates in Eschikon (Canton Zurich), Cadenazzo (Canton Tessin) and Delley (Canton Fribourg). At anthesis, the tassels of the cms hybrids were rigorously checked for presence or absence of pollen, and in the second case controlled self-pollinations were made. From the 20 characterised hybrids, 6 presented a stable sterility across the different locations. Four hybrids were fertile across all locations. Three hybrids produced some anthers but no pollen was released. The rest of the hybrids varied in the degree of anthers and pollen formation from one region to the other, suggesting that some environmental factors do play a role in the reversion of cms to fertility.
A second aim of the first year of field trials was to investigate the abilities and potential use in a Plus-Hybrid system of a large set of modern hybrids. We worked with a large number of modern hybrids available in their cms- and fertile form, collected from the DSP pool but also from several European breeders. During this first season, the main field trials took place in 3 locations in Switzerland: Delley, Canton Freiburg,, Avenches, Canton Freiburg, Lindau-Eschikon, Canton Zürich and one location in the south of France :Montauban
Investigated Parameters: Flowering synchrony, Total grain yield, Number of kernels per surface, 1000- kernel weight. 90% of the samples have been analyzed for 1000-kernel weight and Number of kernels. The rest of the samples is still being counted. Deeper investigations on individual weight of 80 kernels per sample are also running.
The fingerprinting analysis of the hybrids has started. Markers have been tested and selected. SSR-PCRs with these markers are running on the first set of DNA extracted from young etiolated plants.
WP1 partners, involved into task 1.1, decided to carry out ring field experiments in Switzerland, Germany, Bulgaria, France with validated CMS-maize lines :

Maize Ring Test 2006

Maize Ring Test 2006

Within the framework of the European program COEXTRA, several field trials will be carried out in Switzerland, Germany, France and Bulgaria. This will firstly enable an interesting partnership with several research institutes and secondly, enable us to carry out field trials in different geographical places under different environmental conditions. In 2005, field test have already been realized in Switzerland and in France, and the results of this large screening should permit to select the most suitable hybrids that have to be integrated in the experiment in 2006.

Locations & contacts :

- Bulgaria: ABI, Nikolai Christov, Atanas Atanassov
- France: Arvalis, Xavier Foueillassar
- Germany: BBA, Alexandra Hüsken, Joachim Schiemann
- Switzerland: DSP, Magali Munsch, Karl-Heinz Camp & ETH, Christophe Weider, Peter Stamp

Stability of the male sterility under different Environmental conditions

- **Plant material**

In 2006, 10 hybrids selected from the screening realized in 2005, will be tested. These 10 hybrids (see table below) have been selected because they had an unstable behavior in the previous field trials carried out in different places in Switzerland and in France.

General overview of the results 2005

	CMS type	France	Switzerland ZH	Switzerland NE	Switzerland TI
LIM 5	C	S	S	S	S
KWS 2	**S**	**S**	**S**	**S**	**S**
MAD 1	°	S	S	S	S
EUR 1	°	S	S	S	S
DSP 9	T	S	S	S	S
MAD 2	°	S	S	S	S
LIM 4	C	fA	S	S	S
DSP 7	T	fA	S	fA	S
DSP 2	T	X	fA	fA	S
LIM 3	C	fA	fA	fA	fA
SYN 1	°	fA	fA	fA	fA
DSP 8	S	fA	fA	fA	fA
DSP 1	T	fP	S	S	S
KWS 1	**S**	**fP**	**fA**	**fA**	**fA**
SYN 2	°	fA	fP	fP	fP
DSP 5	S	F	fA	fA	fA
LIM 6	C	F	F	F	F
DSP 4	S	F	F	F	F
DSP 6	S	F	F	F	F
DSP 10	C	F	F	F	F

S= **S**terile tassel without anthers

fA= fluctuating* tassel, **A**bsence of pollen

fP= fluctuating tassel, **P**resence of pollen

F= **F**ertile* tassel

STERILE: 6 Hybrids in all the locations

STERILE or FLUCTUATING without pollen: 6 Hybrids

Pollen produced in France but not in Switzerland: 3 hybrids

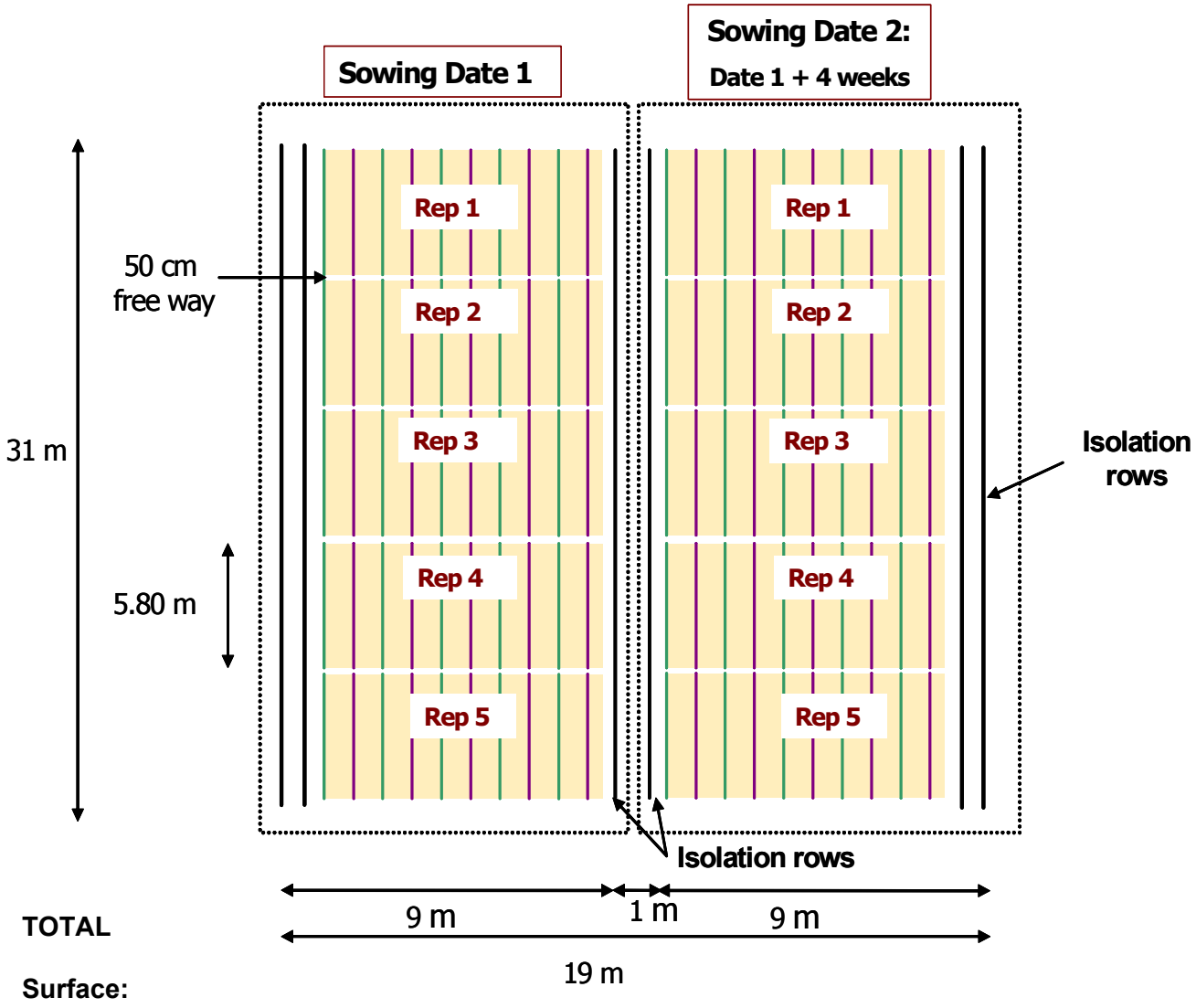
Pollen produced in Switzerland but not in France: 1 Hybrid

FERTILE: 4 Hybrids in all the locations

* fluctuating tassel : anthers only on the secondary bits of the tassel
* Fertile: anthers on the whole tassel
X : Silpro has not been investigated in France
° undeterminated cms type. Is going to be tested thanks to molecular marker

- The selected Hybrids are the following:
  - DSP 1
  - DSP 2
  - DSP 4
  - DSP 5
  - DSP 6
  - DSP 8
  - SYN 1
  - SYN 2
  - LIM 3
  - KWS 1
- The selected Hybrid for the isolation: - Goldenso

- **Test Layout**



TOTAL

Surface:

590 m²

Each CMS-hybrid will be grown in two different sowing dates separated from around four weeks (according to weather conditions) and with five replications per sowing date. The CMS-hybrids will be sown randomly in each replication (see attached Experimental Design). A replication plot consists of 10 rows, corresponding to the **10 tested CMS-hybrids**, 5.80 m long (30 plants separated from 20 cm, and 80 cm between each row). Between each repetition Block, a **way of 50cm** should be free to enable to get through the field.

The aim of the different sowing dates is to expose the plants to different growth conditions within a same climatic zone, and therefore influence the key growth stages. The first sowing date should be around beginning May, and the second one around beginning of June.

The extremity of the two sowing date blocks should be isolated with two rows of a normal fertile hybrid which should be emasculated before flowering to avoid pollen contamination and to give to the first row of the tested cms hybrid a neighbor row. Also between the two sowing date blocks two isolation rows are necessary: one will be sown

together with the first bloc at the first sowing date while the other will be sown with the second bloc at the second sowing date.

- **Amount of seeds necessary for each hybrid**

- For the tested cms hybrid: **30** X **5**repetitions X **2** sowing dates = **300 seeds**
- For the isolation rows: **900 seeds**

- **Research questions**

Under field conditions, do plants produce anthers and pollen?

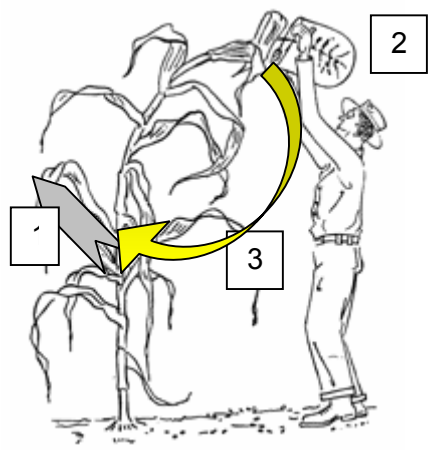
During growth period, the plants will be observed and particular attention will be paid during male flowering time. The **date** of the female flowering as well as the male flowering must be assessed.

Besides, the characteristics of the tassel should be noted. There are three main different types of tassels that can be observed:

- *A **sterile** tassel is not producing any anthers and therefore no pollen,*
- *A **fluctuant** tassel is a tassel producing anthers only on the lateral branches. This type of tassel can produce either no pollen or more or less pollen,*
- *A **fertile** tassel is producing anthers on all the branches. This type of tassel can produce either no pollen or more or less pollen.*

If pollen is produced, is it sterile, or is it able to germinate and fertilize?

The tests that will be carried out with the same care as for the inbreeding pollinations, are firstly dedicated to evaluate the stability of the Cytoplasmic Male Sterility under field conditions. The ear of each plant should be **isolated** before silking! Hybrids whose male flowers produce anthers will be **self pollinated**. The tassel should be isolated the day before the self-pollination.



Steps to follow:

1. **Isolate the ear before silking**
2. **Isolate the tassel the day before the self-pollination**
3. **Self-pollinate and keep the ear isolated**

15 plants per hybrid, per repetition and per sowing date should be self pollinated during the field test. Thus, we will have 75 plants/ hybrid, which will enable a good statistic analysis of the results obtained from the self-pollination.

- **Harvesting and analysis**

The harvesting can start before the kernel maturity has been reached because we are not interested here in the yield. Be careful to harvest each repetition separately and gather the cobs in bags with the name of the hybrid and the number of the repetition.

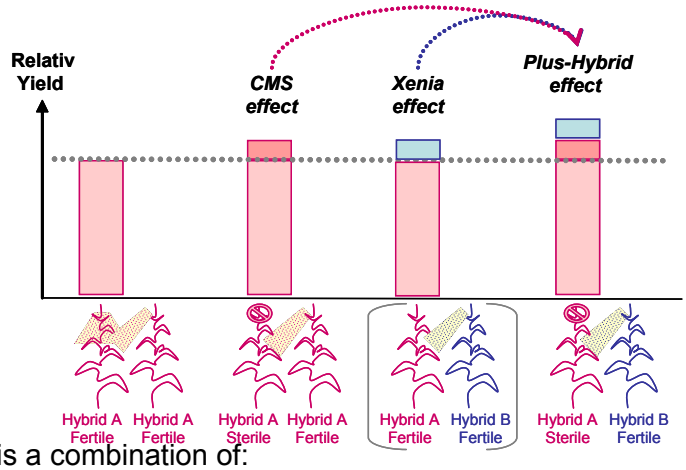
The harvested ears should nevertheless be dried to avoid fungi development and allow a correct stocking. Then the number of kernel on each ear should be assessed according to a protocol that will be sent later on.

Yield improvement through Plus-Hybrid Effect

In the current context where the cultivation of genetically modified crops also in Europe seems to be imminent, Plus-Hybrids can be suggested as a method of containment of the transgene. The spread of transgenic pollen and the 'contamination' of conventional or organic fields in the neighborhood seem to be the main preoccupation. One of the biological approaches for gene flow mitigation could be the containment of the transgene in male sterile plants (no release of transgenic pollen) which are cultivated in mixtures with conventional male fertile bred plants, assuming the fertilization (Feil *et al.* 2003).

"Growing a mixture of a transgenic Cytoplasmic Male Sterile maize hybrid in combination with a lower proportion of a non-transgenic unrelated fertile hybrid as pollen donor for the entire field."

Furthermore, the Plus-Hybrid system is a promising approach for increasing maize grain yield, not only by heterosis, but also by Plus-Hybrid effect.



This Plus-Hybrid effect is a combination of:

- **CMS effect:** difference in grain yield between an isogenic-pollination {A fertile x A ms} and its counterpart {A fertile x A fertile}. This effect has a direct influence on kernel number.
- **Xenia effect:** direct impact from pollen and therefore paternal genetic on grain aspect, composition, weight {A fertile x B fertile}. This effect has a direct influence on the yield parameter Kernel weight.

Two previous PhD studies (Kaeser 2002; Weingartner 2002) focused on the yield results obtained with model hybrids from different world regions. In most of the cases studied, an effectively yield improvement by combination of the gain in yield of CMS- and Xenia-effect up to 20% for the 'best' combination was observed.

The insurance of a high and stable yield is a prerequisite for farmers' acceptance of this type of seed mixtures (with or without GM crops). Breeders must be able to find efficiently "Plus-Hybrids" allowing a real advantage for the farmers in terms of yield. To reach this goal, it is highly essential to determine the optimal combination of already existing hybrids for their suitability within the Plus-Hybrid system. Therefore we screened more than 20 modern high yielding hybrids thanks to test-mother- and test-pollinator-plants in 2005 (3 locations in

Switzerland). This ring test will allow a deeper investigation of the most interesting hybrids we founded out in 2005.

- **Plant material**

According to the results obtained during the season 2005, we selected 5 Hybrids for their putative good pollinating or combining abilities:

- KWS1
- EUR1
- LIM1
- DSP1
- DSP2

Tree others pollinators demonstrating a high pollinating ability in previous trials have also been added as Test-pollinators:

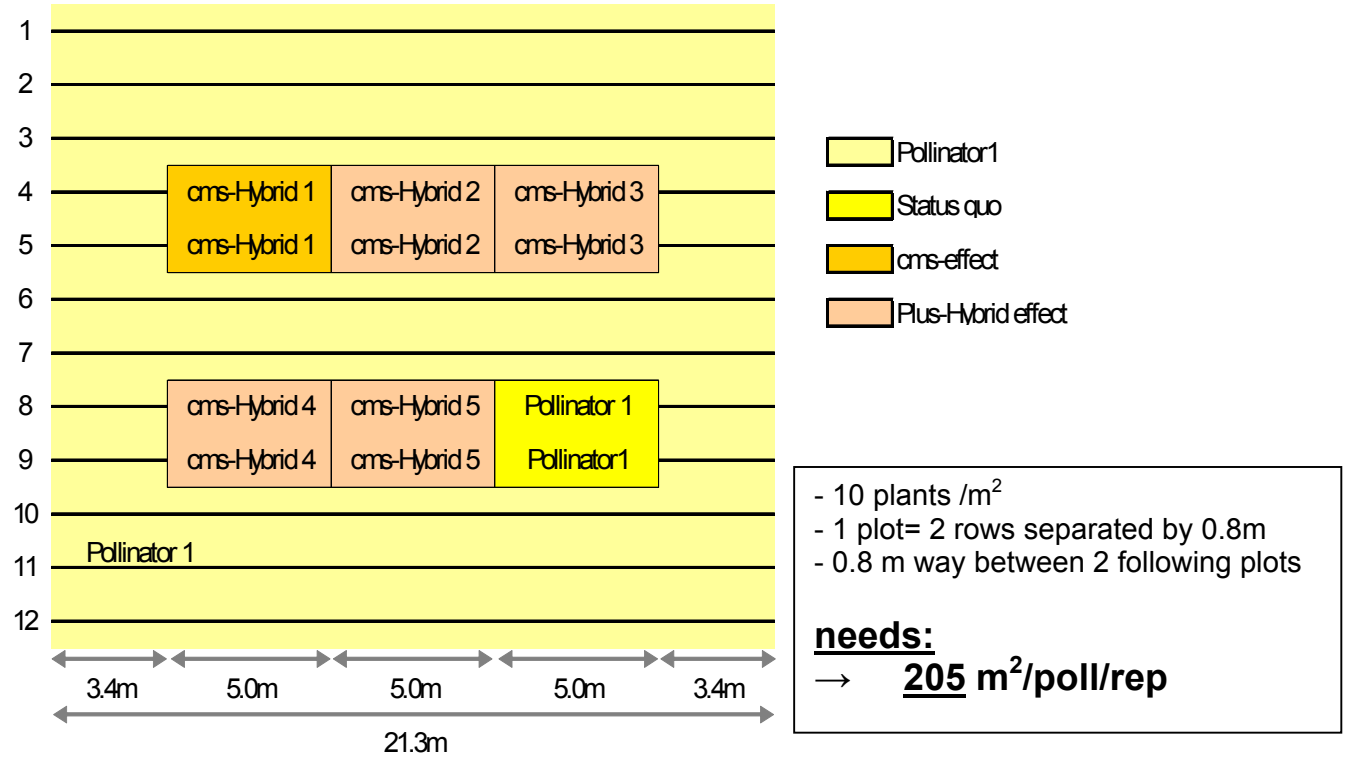
- KWS2
- LIM2
- DSP3

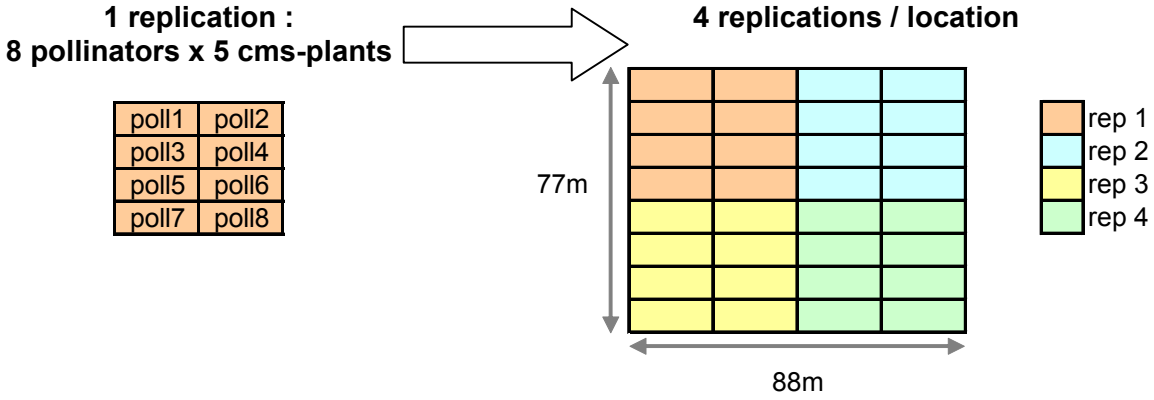
♀ \ ♂		Fertile							
		KWS1	EUR1	LIM1	DSP1	DSP2	KWS2	LIM2	DSP3
sterile	KWS1								
	EUR1								
	LIM1								
	DSP1								
	DSP2								

Plus-Hybrid effect
cms effect

- **Layout:**

The 5 cms-hybrids (2 rows) are randomized within a pollinator block.
The last plot containing the pollinator will be harvested too in order to obtain the reference value: *Status quo:* {Hybrid1 fertile x Hybrid1 fertile}





⇨ **Plus-Hybrid trial : 0.7 ha!**

- **To do :**

- Seeds preparations
- Sowing (~1/2 day depending to the sowing machine)
- Observations: - Number of plants
  - Male and female flowering dates
  - Plant height at flowering
- Harvesting: - Number of plants / parcel at harvest time
  - Number of cobs harvested / parcel
  - Moisture at harvest time
  - Yield (weight harvested)
  - Collect & dry samples for 1000-kernel weight and number of kernels per surface

2.1.1 Cleistogamic rapeseed

The cleistogamous trait has been selected from induced mutagenesis in oilseed rape and patented (Renard and Tanguy, 1997). This trait is controlled, in major part, by one gene (Clg1). There is only restricted literature on cleistogamic rapeseed lines, because INRA Rennes's team is the only one in the world having such kind of biological tool to reduce gene flow. This material has never been given outside the INRA group before the COEXTRA Project Since 1998, several field experiments have been carried on cleistogamous lines of oilseed rape to study their impact on pollination. Presently, lines with a good stability exist and the aim is to verify the impact of cleistogamy both on autogamy and pollen dispersal limitation under several climatic and agricultural conditions or cultivation techniques. Preliminary results from 2004-05 have shown that the main effect is a reduction of pollen dispersal. Another aim is to verify the stability of the cleistogamous trait under several environmental conditions in order to be certain of its interest in various situations. Multilocated field experiments are of prime importance to test stability. From the integration of these results into the GENESYS-Rape model, the benefits of cleistogamy could be considered. During the present experimental year 2 cleistogamous genotypes are being tested in the field at 5 locations (2 in UK, 2 in Germany and 1 in France in WP1 of Co Extra), and under 2 treatments (with or without application of a growth regulator when the growth is restarting at the end of winter). WP1 partners, involved into task 1.1, decided to carry out ring field experiments in Germany, Bulgaria and UK with validated cleistogamic rapeseed lines.

Locations & contacts :

- France: CETIOM, Xavier Pinochet
- Germany: BBA, Alexandra Hüsken, Joachim Schiemann
- UK: NIAB, Don Pendergast

Planned ring experiments:

DON_AGRO	FORMULAIRE D'ENREGISTREMENT EXPERIMENTATION PROTOCOLE	ID : DAGRO_A2_FO_01 *ISO 9001 – 7.2 /7.3/7.5* *BPE – Ch.9.2* *A2 – Revue des protocoles*
Version 1.0	Last modification	15/02/2006
COLZA D'HIVER 2006 B1050010	ETUDE DES LIGNEES CLEISTOGAMES PROJET UE CO EXTRA	C06COE VERSION 1.0 0.1émis le : 05/08/05 V1.0 :26/09/05 V1.1 : 15 FEB 06
protocole emission :		
Pinochet Xavier (CETIOM)		
Contrat	**Projet UE Co-Extra**	

Number of trials and locations			
CETIOM	France	Dijon	1 Trial
BBA	Germany		1 Trial
			1 Trial
NIAB	UK		1 Trial

1 Question and objectives

The aim of this protocol is to check stability of cleistogamic trait during flowering among 2 genetic backgrounds and among 4 locations in UK, Germany and France. This is a contribution to WP1 of the European project Co Extra.

2 Implantation

a. Experimental Design

Dispositif	Split Plot	Blocks Number	4
Factor 1	genotype	Repetitions number	4
Factor 2	Growth regulator	Total number of treatment	-
Controls	Leading CV	Plot area (m²)	-
Harvest	Yes	Harvested area (m²)	around 20 m²

Aviso and Campala are commercial leading cultivars in France. May be ES Astrid and Recital , and Smart could be equivalents for UK and german Markets respectively.

b. Treatments tested

Code	Treatment
1	Aviso / Smart
2	Campala /Recital or ES Astrid
3	Cleisto 1 (17046)
4	Cleisto 2 (16960)
5	Aviso + Growth regulator
6	Campala + Growth regulator
7	Cleisto 1 + Growth regulator
8	Cleisto 2 + Growth regulator

Growth regulator could be a triazole or Parlay applied during autumn or late winter

c. Implantation recommandation

2.1.2 Site choice

Near weather station able to provide rain falls, max and mini daily temperatures, and relative humidity.

2.1.3 Trial management.

Trial will be managed following classical farmers technical practices

3 ChARACTERIZATION of realisation conditions

d. Field and trial Characteristics

- location
- situation (valley, plaine, slop, plateau...)
- type of soil : texture, amont of stones. Give the soil analysis if available.
- Give an estimation of the water avaibiliy in the field.

e. Description to be recorded

At the trial scale give :
- sowing date
- amount of seeds used per m2
- elementary plots surface
- fertilization
- treatements during vegetation specially **fongicides** and growth **regulators** (chemical, date, quantity)
- Harvest date.
- daily weather data : max, min and average t°, hours of sun light, relative humidity, rain falls, wind speed

4 OBSERVATIONS ET CALENDAR

f. List of observations et dates

CALENDAR OF OBSERVATIONS AND SAMPLING MODALITIES				
Date / vegetative Stage required	Things to be done	scale	Reference méthod	Condition of realisation
Autumne or winter (+ eventually during winter end)	Regularity of plant density	plots	G.E. CETIOM 94 – A2	Systématique
Winter beginning. (+ end of winter.)	Fresh aerial weight / m²	trial	French « Réglette Azote »	Depending of the winter conditions.
Autumn	Phoma leaf spots	genotypes	G.E. CETIOM 2005	Facultative / presence of leaf spots with clear variations among cultivars.
Stage C2	Date - stage	genotypes	G.E. CETIOM 94 – A1	Systématique
Stade F1	Date - stade	genotypes	G.E. CETIOM 94 – A1	Systématique
During Flowering	**Each Week Observation of how flowers are open on Iary and IIary stems**	**Plots**	**following INRA's Protocol**	
Stage G4	Height	Plots	G.E. CETIOM 94 – A5 / Rq 1	Systématique
Stage G4	G2 index	genotypes	G.E. CETIOM 2005	
Stage G4	Accidents, diseases or bioagressors occuring on the trial has to be registered, globally if all the trial is concerned.			
maturation Phase	Lodge (1=Absence)	Plots/ Trial	?	Lodge is systematically registered at harvest. If it occur earlier do a first observation and a second one at harvest.
Harvest	weight	plot	-	Systématique

g. Methods to be used for observations

Class 3 for partially open flower

The opening level is noted from 1 to 5. Notation concerns the whole inflorescence. Note 1 corresponds to a completely opened inflorescence similar to a conventional open oilseed rape; note 3 correponds 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. The only matures flowers of the inflorescence are taken into account for notation because i) flower beginning opening looks nearly closed and ii) faded flower looks partly opened in cleistogamous lines which could induce errors in notation. A particular pattern exist: some flowers have grouped 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.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 climatic conditions. For the same reasons, notations will be performed in the morning and in the afternooon of the same day.
In case of variability among plants , take 20 plants per plot.

Accidents, diseases and bioagressors occuring on the trial should be registered, at the trial scale if all the treatments are concerned, and at the plot scale if there are differences among them and if the more susceptible treatment is concerned over 20% of the plants.

h. Sampling analysis

Indiquer les analyses demandées : niveau de prélèvement [1] (traitement, bloc..), la méthode de prélèvement [2], et l'analyse demandée [3] (en précisant, si nécessaire la méthode d'analyse).

Scale of sampling [1]	ANALYSE [3]
Elementary plot	Pure and dry seed weight, Oil content, weight of 1000 seeds .

5 VISITS ON THE TRIAL

	PERIOD	COMMENTS
VISITE 1	During flowering	Possible with Co Extra partners

6 DATA ANALIZES

i. Hypotheses tested

Closed petals and flowers trait is stable during flowering period with a significative difference with classical genotypes.

2.1 Statistic Treatment

Data will be analyze through an ANOVA using SAS software or an equivalent.

7 EXPECTED RESULTS and TIME TABLE

j. Data registration

:

LISTE DES DONNEES ATTENDUES ET DATES				
How	Variable	scale	Unit	avaibility
Excel sheet	Field description and trial management	trial		15 avril
Excel sheet	Number of plants /m2 , homogeneity	plot	Pl. / m² score 1 to 9	15 avril
Excel sheet	DATE C2	plot	Date	15 avril
Excel sheet	DATE F1	plot	Date	20 avril
Excel sheet	height	plot	Cm	1er Juin
Excel sheet	G2 index	plot		15 july
Excel sheet	lodge	plot	Score 1 to 9	
Excel sheet	Harvested weight	plot	Kg	
Excel sheet	Notation during flowering	plot	Scale 1 to 5	15 june